Targeted, semi-targeted and non-targeted screening for drugs in whole blood by UPLC-TOF-MS with data-independent acquisition (DIA)

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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RESUMO

O objetivo deste trabalho foi o de elaborar e validar um novo método de rastreio de fármacos e drogas presentes em amostras de sangue. Rastreios direcionados, semi-direcionados e não-direcionados foram realizados em 55 amostras de sangue proveniente de casos forenses. Todas as matrizes provieram de relatórios medico-legais de casos *antemortem* and *postmortem*.

Ionização por electrospray, no modo positivo, foi utilizada como fonte de iões. A deteção foi feita com base numa aquisição de alta resolução de massa exata realizada por um espectrómetro de massa de tempo-de-voo. Todos os dados foram processados com recurso ao *UNIFI1.7*^{TM} e adquiridos através de uma abordagem de varredura completa, com base numa aquisição independente de dados.

No rastreio direcionado, uma biblioteca comercial de 1030 compostos de relevância fármaco-toxicológica foi adotada. Um total de 231 ocorrências possibilitou a identificação de 103 fármacos, drogas e metabolitos. 119 ocorrências foram confirmadas por análises paralelas de triplo quadrupólo. O método foi avaliado em termos de sensibilidade e especificidade.

Uma biblioteca semi-direcionada contendo 1392 compostos de relevância fármaco-toxicológica não-comuns foi estruturada com base nos resultados do rastreio direcionado. O tempo de retenção foi excluído da informação dos compostos considerados para análise. Os compostos suspeitos foram recolhidos a partir da literatura e de outras fontes científicas/recreativas. No rastreio semi-direcionado foram identificados 15 compostos suspeitos, distribuídos por 21 ocorrências. A presença de 3 dos suspeitos foi confirmada pela análise das respetivas soluções padrão. A capacidade de previsão do padrão de fragmentação por parte do *UNIFI1.7*^{TM} foi avaliada. Uma ocorrência suspeita provou ter elevada probabilidade de corresponder a uma “designer drug”.

Um rastreio não-direcionado adquiriu a massa exacta correspondente a ocorrências desconhecidas. Uma pesquisa invertida foi aplicada às massas exactas através da predição de fórmula molecular por parte do *UNIFI1.7*^{TM}. As estruturas moleculares de um composto endógeno e de um composto de relevância fármaco-toxicológica foram elucidadas.

Palavras-chave: rastreio direcionado, rastreio semi-direcionado, rastreio não-direcionado, espectrometria de massa de tempo-de-voo, alta resolução, massa exata, aquisição independente de data, fármacos, drogas de abuso, “designer drugs”.

ABSTRACT

The objective of this work was to formulate and validate a new screening method for legal and illegal drugs, which presence is in whole blood. Targeted, semi-targeted and non-targeted screenings in 55 blood samples from forensic cases. All matrixes were provided from medico-legal *antemortem* and *postmortem* case reports.

Electrospray ionization (ESI), in positive mode, was used as ion source. Detection was based on a high resolution accurate mass (HR/AM) acquisition provided by time-of-flight mass spectrometry (TOF/MS). All data was processed using *UNIFI1.7™* and acquired through a full scan approach based on data-independent acquisition (DIA).

In targeted screening, a commercially available library containing 1030 common pharmaco-toxicological relevant compounds (PTRCs) was adopted. A total of 231 hits made possible the identification of 103 drugs and metabolites. 119 hits were confirmed by parallel analysis with triple quadrupole (MS/MS). The method was evaluated in terms of sensitivity and specificity.

A semi-targeted library containing 1392 non-common PTRCs was assembled, excluding retention time (RT) from the information of the compounds considered for analysis. Suspects were gathered from literature and other scientific/recreational sources. Semi-targeted screening identified 15 suspect compounds, distributed by 21 hits. 3 compounds were confirmed by the respective pure standards. *UNIFI1.7™* capacity of fragmentation pattern prediction was evaluated. A highly probable designer drug hit was semi-targeted identified.

A non-targeted screening acquired exact mass of unknown hits. A reverse search was applied in AMs by prediction of molecular formula by *UNIFI1.7™*. The molecular structures of one endogenous compound and one PTRC were elucidated.

Key-words: targeted screening, semi-targeted screening, non-targeted screening, TOF/MS, HR/AM, DIA, PTRC, designer drug.

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ABBREVIATIONS

Abbreviations	Description	Units
λ	Structure factor of the column packing material	
Δ^9-THC	Tetrahydrocannabinol	
2-AI	2-aminoindane	
4-AcO-DMT	O-acetylpsilocin	
6-MAM	6-Monoacetylmorphine	
α-PPP	α-pyrrolidinopropiophenone	
A	Multiple flow paths constant	
AC	Alternate Current	
ACN	Acetonitrile	
ADHD	Attention-Deficit Hyperactivity Disorder	
AM	Accurate Mass	
APCI	Atmospheric Pressure Chemical Ionization	
B	Longitudinal diffusion constant	
BAMBS	Beta-agonists Asthma Drugs of abuse Benzodiazepines Special ("Beta-agonister Astma Misbrugsstoffer Benzodiazepiner Specielle")	
BZP	Benzylpiperazine	
C	Finite equilibration between phases	
CE	Capillary Electrophoresis	

Abbreviations	Description	Units
CID	Collision Induced Dissociation	
CNS	Central Nervous System	
CHP	Cyclohexylphenols	
CP	Confirmed Positive	
d	Distance	m
Da	dalton	
DAD	Diode-Array Detection	
DIA	Data Independent Acquisition	
DDA	Data Dependent Acquisition	
dp	Particle diameter	µm
D_M	Diffusion coefficient	
DUID	Driving Under the Influence of Drugs	
EI	Electron Impact	
ESI	Electrospray Ionization	
EPI	Enhanced Product Ion	
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction	
FN	False Negative	
FP	False Positive	
FWHM	Full Width Half Maximum	
GUS	General Unknown Screenings (synonym of **STA**)	
H	(HEPT) Height equivalent to a theoretical plate	µm
HE	High Energy function	
HPLC	High Performance Liquid Chromatography	
HR/AM	High Resolution Accurate Mass	
HR/MS	High Resolution Mass Spectrometry	
(HR)MS	High Resolution Mass Spectrometer	
HSS	Hollow Structure Section	
IS	Internal Standard	

Abbreviations	Description	Units
IMS	Ion Mobility Spectroscopy	
IT	Ion-Trap	
K	Retention factor	
k	Unit less constant	
LC	Liquid Chromatography	
LC-IT/MS	Liquid Chromatography Ion-Trap Mass Spectrometry	
LC-MS	Liquid Chromatography Mass Spectrometry	
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry	
LE	Low Energy function	
LIT	Linear Ion Trap	
LIMS	Laboratory Information Management System	
LLE	Liquid-Liquid Extraction	
LOD	Limit of Detection	
LOF	Limit of Fragmentation	
LOI	Limited of Identification	
LSD	Lysergic Acid Diethylamide	
LTC	Lower therapeutic concentration	mg/Kg
m	mass	$g.mol^{-1}$
MA	Mass accuracy	
ME	Matrix Effect	
MeOH	Methanol	
MDMA	Methylenedioxymethamphetamine (ecstasy)	
mDa	Milidalton	
MRM	Multiple Reaction Monitoring	
MS	Mass spectrometer or Mass spectroscopy	
MS/MS	Tandem Mass Spectrometry	
MS^e	High resolution mass spectrometry (synonym of **DIA**)	
MS^n	Multiple fragmentation energy mass spectrometry	

MW	Molecular Weight	
Abbreviations	**Description**	**Units**
m/z	Mass-to-charge ratio	
N	Number of plates	
NIST	National Institute of Standards and Technology	
NCBI	National Center for Biotechnology Information	
NSAID	Non-Steroidal Anti-Inflammatory Drug	
PCP	Phencyclidine	
PhPL	Phospholipids	
PTRC	Pharmaco-Toxicologically Relevant Compounds	
PP	Phenylpiperazine	
PPT	Protein Precipitation	
q	Individual charge of a particle	C
Q	Quadrupole	
QIT	Quadrupole Ion Trap	
Qtrap	Hybrid triple quadrupole linear ion trap	
RKA	Section of Forensic Chemistry, Department of Forensic Medicine, University Of Copenhagen	
RP	Resolving Power	
rpm	rotations per minute	
RT	Retention Time	
S/N	Signal-to-noise ratio	
sMRM	scheduled Multiple Reaction Monotoring	
SPE	Solid-Phase Extraction	
SSRI	Selective Serotonin Reuptake Inhibitor	
STA	Systematic Toxic Analysis (synonym of **GUS**)	
t	Time	s
TIC	Total Ion Chromatogram	
TCA	Tricyclic Antidepressant	

TOF	Time-of-Flight	
Abbreviations	**Description**	**Units**
TOF/MS	Time-of-Flight Mass Spectrometry	
TP	Transformation Product	
U	Electrical potential difference	V
UPLC	Ultra-Performance Liquid Chromatography	
UP	*UNIFI1.7™* Positive	
UV	Ultra-Violet	
UNDOC	United Nations Office on Drugs and Crime	
u_x	Linear flow rate	$m.s^{-1}$
v	Velocity	$m.s^{-1}$
XIC	Extracted Ion chromatogram	

1 INTRODUCTION

1.1 RKA

This project was carried out in the Section of Forensic Chemistry, Department of Forensic Medicine, University of Copenhagen or simply abbreviated RKA**.**

The department provides a wide offer of toxicological analysis. As such, it undergoes about 600 medico-legal autopsies per year, analyzes about 1800 driving under the influence of drugs (DUID) cases, in complementarity to around 10000 driving under the influence of alcohol cases.

Additionally, the department examines around 1000 police drug related seizures and about 400 violence and rape cases.

The geographical area covered by RKA comprehends eastern Denmark (Zealand, surrounding islands and Bornholm) highlighted in dark green in **Figure 1.1**.

The population served by this forensic department is of approximately 2.4 million, from a total of 5.6 million that represent all of Denmark and its dependencies.

Figure 1.1 *Map of Denmark.*

The biological matrixes toxicologically analyzed in a routinely basis are blood, urine, muscle and hair samples.

It is expected that a toxicological analysis covers all compounds and to achieve the closest results to that premise a conjugation of methods is performed. As a standard screening setup, two methods based on liquid chromatography time-of-flight mass

spectrometry (LC-TOF-MS) are coordinated to provide unambiguous detection and identification of pharmaco-toxicologically relevant compounds (PTRC).

With respect to whole blood samples, the 5 validated and routinely used methods for screening are present in **Figure 1.2**'s diagram.

Figure 1.2 *Outline of five different methods employed for screening and quantification purposes in toxicological analysis of whole blood samples.*

The green segment of **Figure 1.2** is what was meant to be upgraded in the project. At first instance, the main improvements were the reduction of the needed sample volume (pre-sample preparation) from 200 µL to 100 µL; the increase of compounds in the targeted database, from 400 to 1030 basic drugs; and the implementation of semi and non-targeted screening approaches.

1.2 Systematic Toxicological Analysis

Systematic toxicological analysis (STA) is the general search for pharmaco-toxicologically relevant compounds in a biological sample. The array of matrixes comprises human blood, urine, organ tissue and hair. The screening is performed without prior analytical information concerning the presence and category of the xenobiotic poison [1-5]. Due to the enormous number of possible PTRCs and their metabolites (most of the times present in very low concentrations) and because of the complexity of the matrixes, general unknown screening (GUS) is credited as one of the most difficult tasks in the field of analytical chemistry [1].

It is possible to categorize PTRCs in 4 groups: toxic gases, volatile substances, metal ions and, the largest category (and the one of relevance for this thesis), organic compounds with low volatility. To the latter subdivision it can be appointed: pesticides, chemical reagents, alkaloids and drugs for therapeutic uses and purposely synthesized or traded with illegal intentions [1].

This project´s targeted screening is restricted to scan for small molecules pertaining to the drugs (of abuse/legal) sub-category. Complementarily, the semi-targeted and non-targeted approaches provide a screening platform for all types of organic compounds with low volatility.

Today, STA of non-volatile organic small molecules starts with an appropriate and fast sample preparation for wide extraction of as many compounds as possible from the complex and frequently complicated matrixes [1]. Most remarkably, the analytical process that follows has suffered a great degree of alteration in the past few decades [1-5].

Nowadays, forensic samples will initially be directed to immunochemical techniques, including non-instrumental on-site (performed by police officers) and instrumental format. This initial stage is not limited to immunoassays, being complemented by more capable screening techniques employing gas chromatography with mass spectrometry (GC-MS) and high performance liquid chromatography with diode array detector (LC-DAD) [5] and more recently LC-MS [1-6]. These screening procedures mainly concern is to provide a yes or no, rapid-response based on threshold concentration; or the provision of more elaborated qualitative and even semi-quantitative information, in the case of chromatography-detector methods. Especially when employed LC-MS methods [6].

In the case of a positive result in the screening step, an additional 2nd stage is employed were the same sample is subjected to a confirmatory method that provides a higher level of confidence in the result. Generally, the additional selective confirmation and quantification is carried out by GC-MS or LC-MS/MS [6].

From the conjugation of high sensitivity and selectivity of their MS detectors and the availability of extensive libraries of standardized electron impact (EI) mass spectra, GC-MS has long been considered as the gold standard for STA [5].

Nevertheless, the application of the method above is confined to the screening of volatile, thermally stable and non-polar compounds. For instance, analysis of urine (that along with blood is the most relevant toxicological matrix) is impaired due to its richness in polar metabolites [2].

Indeed, an additional and related disadvantage of GC-MS is the time-consuming and labor-intensive compound derivatization needed for analysis of low-volatile and polar analytes [2]. The previously stated HPLC-DAD may alleviate the referred GC-MS constrains, however at the cost of detection power, as DAD is not as specific as MS detectors. Additionally sensitivity may also be compromised as DAD fails to detect molecules with no or little UV (ultra-violet) absorbance [5]. To troubleshoot this impasse, in the last decade several advances have been made in liquid chromatography (LC) combined with mass spectroscopy (LC-MS or/and LC-MS-MS) and with convenient ions sources such as electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI) [1].

Although LC-MS GUS has become well accepted as a confirmatory tool, screening methods employing this instrument are not as established as the two previous methods [5]. Several methodologies have been published such as single-stage MS detection (LC-MS)

combined with reference libraries [7]; ion trap (LC-IT/MS) [4] and tandem mass spectrometry (LC-MS/MS) [8]. However no single technique has conciliated sufficient specificity and the capability of screening for complete unknown chemicals. The majority of LC-MS approaches simply targets chromatographic and spectral information against a panel of compounds and their respective nominal masses. The reduced number of commercial libraries and their incompatibility for instruments of different brands, further complicates targeted screening. Additionally, one of the limitations of using nominal mass is that not enough resolving power is provided for an efficient non-targeted screening [1, 2, 5].

In an attempt to address the issue, time-of-flight/mass spectroscopy (TOF/MS) technology have been developed in order to provide compatibility of this instrument with the STA context. The interest on this method derives from the TOF/MS capability to assign mass to charge ratio (m/z) to four decimal places. Additionally and very importantly, for the non-targeted screening, the analyzer/detector has the ability to provide m/z values to unknown compounds with a mass error interval of less than 5 millidalton (mDa) [9]. This provides the possibility to assign a unique elemental molecular formula based on the mass sufficiency of each constituent atom and on the pattern between the isotopes of the protonated ion present in the spectrum [5].

With the highlighted properties, methods like the one developed in the this thesis, which employed instrumentation and software present in **Figure 1.3**, are expected to provide systematic toxicological analysis (same as general unknown screening) with a compound identification confidence and selectivity that not only rivals but surpasses the other "mainstream" LC-MS screening methods.

Figure 1.3 *UPLC-qTOF/MS.*

1.3 Pharmaco-Toxicologically Relevant Compounds

PTRCs comprise all the pertinent small molecules that should be identified in a general unknown screening. The less ambiguous categorization format of these compounds is based on their presence in the annual reports of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [10] and simply the amount of knowledge available concerning their chemical properties.

As for this, three categories can be delineated: Pharmaceuticals, Drugs of abuse (catalogued) and Designer drugs (some catalogued but the vast majority absent from the EMCDDA "black list").

For agreement with this project, the three categories were converged into two, where the differentiation criterion was the compound's presence/absence in the library used for targeted screening.

1.3.1 Pharmaceuticals and drugs of abuse

1.3.1.1 Pharmaceuticals

Most of compounds detected in STA correspond to medical prescribed and over-the-counter drugs. Their molecular ions and respective metabolites corresponded to the bulk of the positive hits in this thesis.

A short list of commonly identified compounds in a STA, in the forensic context, is present in **Table 1.1**.

Table 1.1 *Common pharmaceuticals detected in a STA.*

Pharmaceutical	Toxicological information
Paracetamol	Widely used over-the-counter analgesic and antipyretic. Deliberate or accidental overdoses are not uncommon, probably due to wide availability [11].
Diazepam	A benzodiazepine with anticonvulsant, anxiolytic, sedative, muscle relaxant, and amnesic properties and a long duration of action [12]. Although not usually fatal when taken alone, overdose can ultimately lead to a state of comma [13].
Citalopram/Escitalopram	Antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) [14]. Overdose deaths have been reported. It is the most dangerous of SSRIs in overdose [15].
Chlordiazepoxide	A benzodiazepine with sedative/hypnotic properties. The first benzodiazepine discovered by serendipity. The drug is frequently involved in drug intoxication, including overdose [16].
Zuclopenthixol	Potent neuroleptic drug of the thioxanthene class. Despite rare, occurrence of fatalities have been reported due to neuroleptic malignant syndrome, induced from over dosage [17].

Additionally, as an example of a toxicological relevant pharmaceutical, quetiapine is hereby illustrated (molecular structure displayed in **Figure 1.4**) in function of its importance for this thesis.

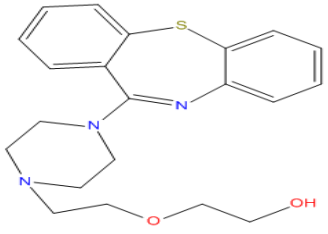

Figure 1.4 *Quetiapine chemical structure.*

Quetiapine possesses antipsychotic properties normally prescribed in the treatment of schizophrenia [18] and

bipolar disorder [19]. Additionally it acts as an antidepressant, regularly used in the treatment of major depressive disorder. Its major active metabolite is 7-OH-quetiapine. [20].

Acute over dosage generally translates in sedation and tachycardia. However, cardiac arrhythmia, coma and death have been reported [21].

1.3.1.2 Common drugs of abuse

The other group of compounds screened for are drugs which are used for a nontherapeutic effect, which may lead to organ damage, addiction, and disturbed patterns of behavior. The most common are alcohol, nicotine, marijuana [22]. These were not taken into account in this method.

The most relevant common “street” drugs considered for this screening method were: opiates, opioids and cocaine (see **Table 1.2**) [22].

Table 1.2 *Example of common drugs of abuse.*

Class	Example	Toxicological information
Opiates	**Morphine**	Main psychoactive chemical in opium and the gold standard in commercialized analgesics [23]. It acts directly in the central nervous system (CNS). Overdose cause asphyxia and death by respiratory depression [24].
	Codeine	Methylated morphine which occurs naturally and has a well-defined and regulated range of therapeutic applications [23]. Deaths by overdose have been reported due to its potential misusage for recreational purposes [25].
Opioids	**Heroin**	Diacetylmorphine with no direct psychoactive effects. It is its metabolization, in the brain, into 6-monoacetylmorphine (6-MAM) and morphine, that induces the acute transcendent state of euphoria [26].
	Methadone	Therapeutically used as an analgesic and anti-addictive in opioid detoxification [27]. Deaths of patients using prescribed methadone are normally attributable to combined intake with alcohol and/or other drugs [28].
Tropane alkaloids	**Cocaine**	Powerful CNS stimulant. Biologically it functions as a SSRI and as a nonspecific voltage gated sodium channel blocker, which in turn causes the compound to produce anesthesia at low doses [29]. Cocaine intake in high doses instigates the compound´s blocking effect on cardiac sodium channels and overdose may result in sudden cardiac death [30].

1.3.2 Designer drugs

1.3.2.1 Designer drugs of abuse

Per definition, designer drugs are synthetic compounds that do not occur naturally, which is also a characteristic of most pharmaceuticals and widely legislated drugs of abuse.

However the structure backbone of all three categories is often based on the structures of naturally occurring compounds [10].

What is distinctive in designer drugs is that they are a result of a chemical adulteration of other drugs already known to be on the illicit marker circuit (see **Figure 1.5** to **Figure 1.12**). There are 8 principal classes of designer drugs: aminoindanes, synthetic cathinones, phenethylamines, phenylcyclohexylamines, piperazines, pyrrolidinophenones, synthetic cannabinoids, tryptamines. Descriptive diagrams for each group are presented in next section: "Designer Drug Classification**"** [31].

The development and synthesis of novel drugs is either performed (inadvertently) by the pharmaceutical industry in the search for new pharmaceuticals or by illegal laboratories and vendors that intentionally seek to circumvent the legislation, as new drugs *per* definition are legal until they are classified illegal [10, 31].

The main social impact of these "new" drugs is their availability on the Internet, sold as "legal highs", "spices", or "bath salts" in order to conceal their illegal nature [32-34]. Most importantly, these drugs represent a risk to abusers as there are numerous reports of accidental overdoses after ingesting tablets or powders with different content and/or repercussions not anticipated by the consumer [35-43].

As an example, the effects of synthetic cannabinoids (**Figure 1.11**) are largely similar to those of tetrahydrocannabinol (Δ^9-THC), as they have affinity for the cannabinoid receptors, CB_1 and CB_2 [44]. However, the over-dosage risk increases with the designer drug intake due to their enhanced affinity with the receptors, and potency, when compared with natural occurring cannabis [45]. Additionally, the fact that the content of synthetic cannabinoids in herbal mixtures often differs from batch to batch increasing their clinical unpredictability, which may ultimately lead to over dosages [46].

In GUS context, the problem rises as in general these drugs cannot be detected in common drug screening assays, mainly because the respective pure standards are not of easily availability (if ever synthetized). The problem is aggravated by the fact that, as substances become known and consequently banned, new analogues replace them making identification of designer drugs a challenge. This thesis will ultimately function as a new attempt to screen for these substances.

1.3.2.2 Designer drug classification

The hereby displayed designer drug classification provided the foundations for the in-house assembled library in the ambit of the semi-targeted screening stage of this project (see **section 3.2.1)**.

Aminoindanes

Nine compounds are known to be part of this class and all of them derive from the 2-aminoindane (2-AI) structure. The two most relevant designer drugs of this class are illustrated in **Figure 1.5** along with the referred precursor drug [47].

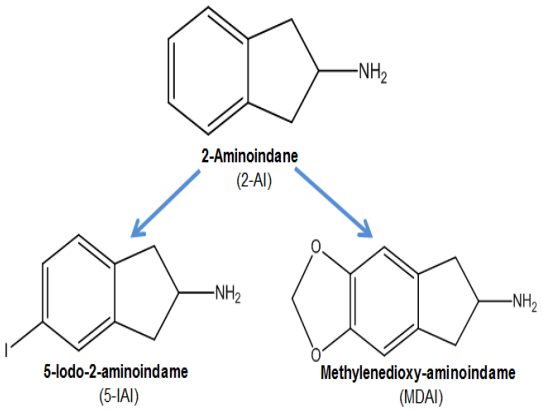

Figure 1.5 *Aminoindane based designer drugs.*

The toxicological effect of compounds of this class has been reported to be entactogenic (empathogenic). It is very similar to the distinctive emotional (feeling of empathy) and social effect that usually follows ecstasy (MDMA) intake [47].

Synthetic cathinones

Officially reports state that there are 44 synthetically derivative compounds from cathinone. The small structure of this well documented illicit drug provides a good platform for chemical adulteration [48].

Synthetic cathinones are frequently found in products sold in powder, pill or capsule and labeled as “plant food”, “bath salts” or “glass cleaner”. The “new age” synthetic drugs of this class are: mephedrone (*“m-cat”* or *“miaow”*) and methylone (*“top cat”*); both present in **Figure 1.6** with the respective base structural compound [48].

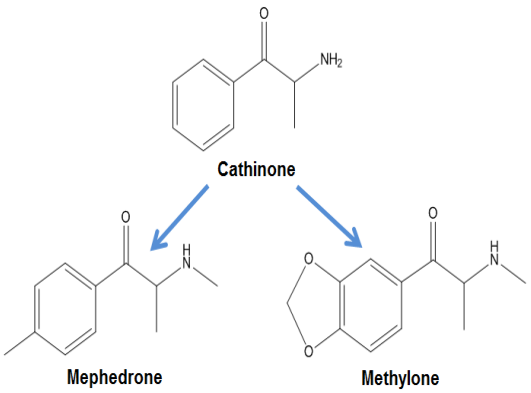

Figure 1.6 *Cathinone based designer drugs.*

The two drugs are usually available as white or brown powders or in the form of pills that are often sold as ecstasy, as their psychoactive effects mimic those of a phenethylamine. Mephedrone is commonly nasally insufflated, injected, ingested by swallowing a powder wrapped in paper (“bombing”), or mixed in a drink [48].

Phenethylamines

Comprising more than 100 compounds, this category of designer dugs is probably the largest and the most relevant in regard to drug abuse cases, as this class is the most often encountered in forensic cases (RKA context) [6].

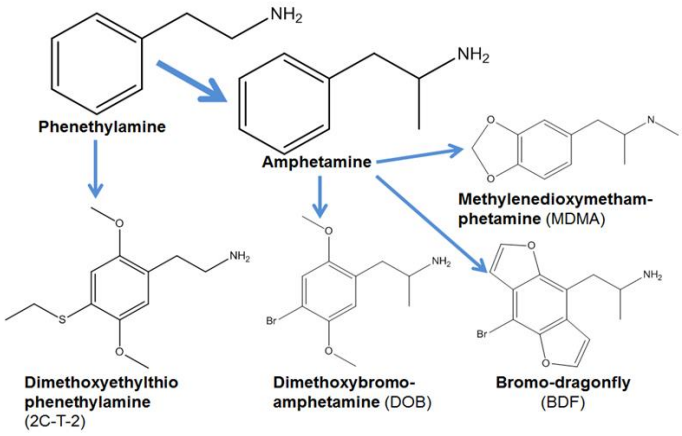

Figure 1.7 *Phenethylamine/Amphetamine based designer drugs.*

Compounds of this class are well known for their stimulating and hallucinogenic psychoactive effects [47].

The backbone of this category is shared by both phenethylamine and amphetamine, whose structures are present in **Figure 1.7**, along with some of their most relevant derivatives [47].

Phenylcyclohexylamines

The backbone of this class is the molecular structure of phenylcyclohexilamine. Phencyclidine (PCP) and ketamine (illegal and pharmaceutical, respectively) are the most relevant phenylcyclohexanamine derivatives [2]. The referred compounds and other of relevance are present in **Figure 1.8** [31].

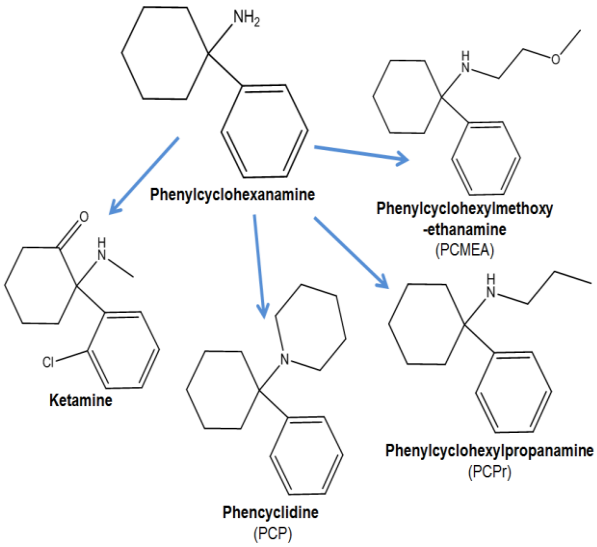

Figure 1.8 *Phenylcyclohexilamine based designer drugs.*

This category comprises around 55 drugs but only 8 have been reported in drug abuse related cases. Designer drugs of this class interact with many different receptor systems; however the predominant psychotropic effects are the anesthetic and hallucinogenic [31].

Piperazines

Benzylpiperazine (BZP) and Phenylpiperazine (PP) are the precursor small molecules behind the 11 designer drugs of this class reported so far. An example of structure adulteration for each parent compound is provided in **Figure 1.9** [49].

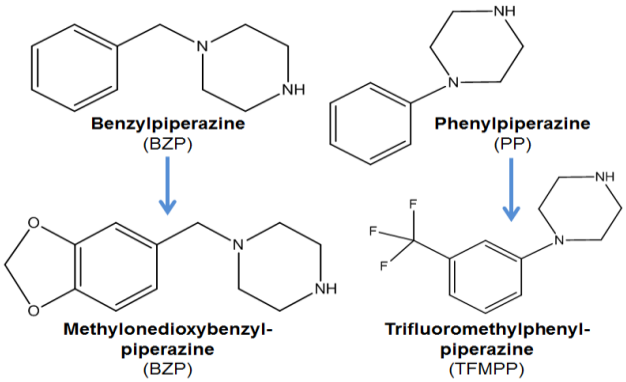

Figure 1.9 *Piperazine based designer drugs.*

Their stimulating and hallucinogenic psychotropic effects mimic those of the phenethylamine/amphetamine class. Indeed, piperazines are often encountered in MDMA tablets, either with sole chemical activity or in combination with ecstasy [49].

Pyrrolidinophenones

The compound that functions as base for this class, alpha-pyrrolidinopropiophenone (α-PPP) is itself a synthetic cathinone. However, because at least 12 different drugs of abuse have been described as direct chemical adulterations of α-PPP, a sole class may be considered. **Figure 1.10** illustrates the parent drug and the two most pertinent pyrrolidinophenone based designer drugs. The *para* position of the phenyl ring and the length of the alkyl chain (up to 6 carbons) are the unique structure substitution sites, reported so far [50].

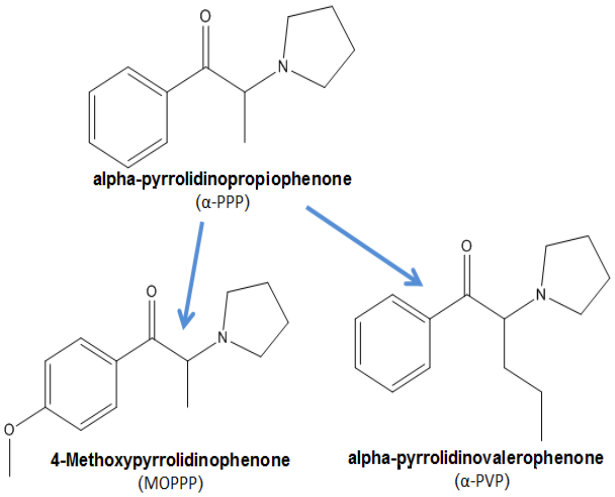

Figure 1.10 *Pyrrolidinophenones based designer drugs.*

From the twelve drugs listed to be part of this class, all demonstrated to have the same stimulating and euphoric effects as phenethylamines. Likewise, interferences with the norepinephrine and dopamine neurotransmitter systems mediate the activity of psychotropic compounds of this class of designer drugs [50].

Synthetic cannabinoids

The chemical activity of drugs pertaining to this class is referenced to be fully or partially agonist for the same receptors targeted by Δ^9-THC. However, the latter and its synthetic counterparts seldom share the same core structure (examples presented in **Figure 1.11**); and accordingly to the molecular shape, 4 sub-categories can be delineated [31]:

- Δ^9-THC analogues, classical cannabinoids (e.g. HU-211) [31];
- "JWH" compounds, comprising naphthoylindole, naphthoylpurrole, or naphthalene structure (e.g. JWH-007) [51];
- Cyclohexylphenols, e.g. CP55,940, inadvertently synthetized by Pfizer [31];
- All other compounds that do not fit into the structural description mentioned in the previous groups but with similar psychoactive activity (e.g. Pravadoline) [31].

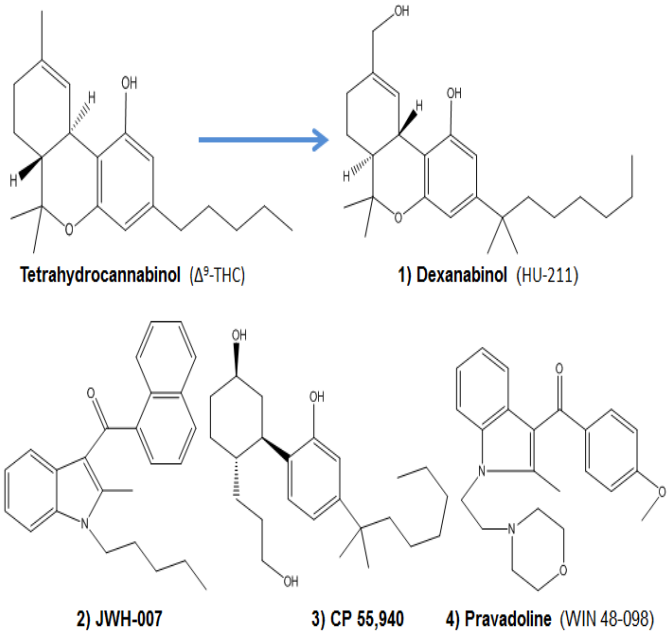

Figure 1.11 *Synthetic cannabinoids designer drugs.*

Tryptamines

Although tryptamine itself (an endogenous neurotransmitter) does not hold a drug of abuse potential, its derivatives (25 different drugs reported so far) had proved otherwise. In this class about half of the compounds naturally occur in plants, fungi or animals. In **Figure 1.12** 4 examples of non-synthetic tryptamine based designer drugs (DMT, Psilocybin, 5-MeO-DMT and Butofenine) and one last, 5-MeO-DIPT, of synthetic origin are present [52].

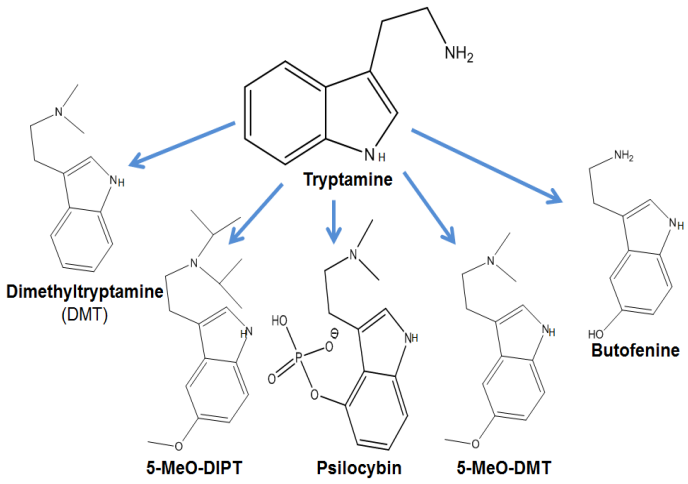

Figure 1.12 *Tryptamine derivate designer drugs.*

Most of the tryptamine derivative drugs are substituted at the aromatic ring and/or at the amine positioned in the end of the aliphatic chain [52].

1.4 Method theory

The diagram of **Figure 1.13** demonstrates the comprehensiveness of the combination of instruments and techniques required for the development of the projected screening method.

Each topic is individualized and theoretically stressed along this chapter.

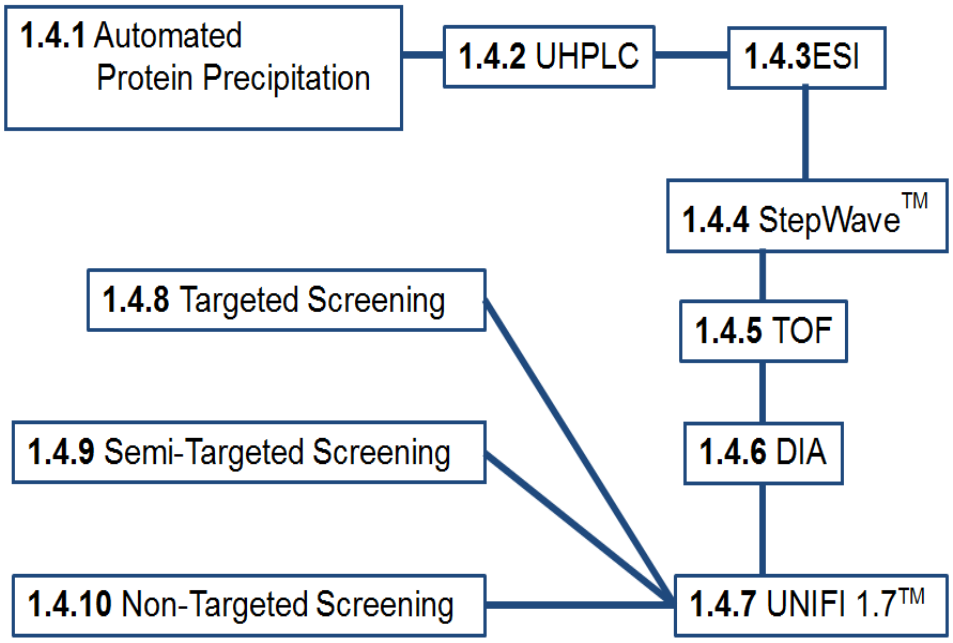

Figure 1.13 *Summary diagram of instrumentation, software and methods adopted in this thesis.*

1.4.1 Sample preparation- Automated protein precipitation

As referred, a STA covers an array of different biological matrixes, and considering those that *a priori* are in the liquid phase, it would be inappropriate to directly inject them into a GC or LC system (in the case of a GUS that employs chromatography) [53]. This inconvenient is justified by the composition of the biological samples, where high contents of phospholipids (PhPL) are a major source of matrix effects (ME) in ESI [54]. Other interfering compounds are present in these matrixes such as salts and endogenous small molecules, irrelevant for the analysis, which diminish separation efficiency. However, the most relevant interference presence in biological matrixes is its protein content [53, 55].

Especially when working with plasma or whole blood, both with high amount of proteins, liquid chromatography columns are prone to wear because of occurrence of protein precipitation in the mobile phase [53, 56]. Additionally, for analyte identification and quantification, it is needed to intermit the protein-drug binding so the compounds of interest can be extracted for further analysis [57].

For this reason, sample preparation is an essential part of GUS in biological matrixes, where the focus is to isolate analytes of interest from the unwanted remains of biological material. Today, the conventional techniques for the effect are: protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) [53, 57].

PPT, the technique adopted for this experiment, is the simplest, fastest and in controlled conditions, the most effective of the three options for STAs [58].

There are four different variants of this sample preparation; either by addition of organic solvent, acid, salt or metallic ions. Nevertheless, the first is the most widely employed [4, 5] and was the one adopted in this project.

Acetone, acetonitrile (ACN), and methanol (MeOH) are common organic solvents used for PPT [4]. The three are miscible with water, capable of lowering the dielectric constant of the solution, which results in attraction between macromolecular structures, and consequently, electrostatic interaction between proteins will augment. Supplementary, by displacement of water molecules, these solvents minimize hydrophobic interactions of the proteins which further increase the predominance of electrostatic interactions between these macromolecules, ultimately causing their aggregation. Then after a centrifugal step, clear distinction between a precipitated protein layer below an organic phase is achievable [53, 56, 57].

LLE and SPE are accounted to normally provide cleaner extracts than PPT [58]. However under automated conditions, this disadvantage is minimized to the point that the latter yield better analyte extraction than its counterparts [59].

In this work a Tecan Freedoom Evo 200™, a fully-automated robot represented in **Figure 1.14**, was used for the performance of all liquid handling required in a PPT sample preparation. In addition to the drastic decrease of time consumption and labor intensity, this fully-automated liquid handling also increases the extraction efficiency and the pipetting reproducibility, since the robot perform the task in the same way, every time.

Figure 1.14 *Tecan Freedom EVO 200™. **1**, Robotic manipulator arm; **2**, carrier for 96-well plates; **3**, weighing-machine; **4**, liquid Handling arm; **5**, washing and tips waste station; **6**, racks with disposable tips; **7**, carrier for tubes; **8**, shaking platform; **9**, carrier for troughs; **10**, plate sealer; **11**, centrifuge; **12**, evaporator; **13**, vacuum station [59].*

For further details on the operation of the robot, see **section 2.3.1**.

1.4.2 Ultra-Performance Liquid Chromatography

Chromatography is the method of choice in bio-analytical laboratories. To satisfy the search for a fast and controlled analyte separation, new trends of liquid chromatography have been employed in recent years. They involve monolith technology, fused core columns, high temperature liquid chromatography and ultra-performance liquid chromatography (UPLC). Recently, the latter has become a wide-spread separation option with focus on fast and sensitive assays, and was the selected chromatographic technique to tandem the analyzer adopted for this thesis [58].

UPLC is a part of the liquid chromatography category. This technology provide analytical access to about 80% of the chemical universe, unreachable by GC and indirectly is also the main responsible for the phenomenal growth and interest in mass spectroscopy [60].

In its simplest form, LC relies on the ability to reproduce, with great precision, competing interactions between analytes, the eluent (the mobile phase) and a bed of packed particles (the stationary phase) [60].

Over the past few decades, technological developments allowed better sealing and pumps, able to hold higher pressures, enabling UPLC columns to be packed with sub-2-micron (µm) particles [58].

Accordingly to van der Deemter, Giddings and Knox theories, efficiency expressed as the height equivalent to a theoretical plate (H) is proportional to particle size squared. Hence, it is inversely proportional to the particle diameter (d_p), as expressed in **equation (1.1)** [58].

Therefore UPLC columns, now with the possibility of being packed with particles with a diameter down to 1.7 µm, provide a well suited solution in the quest to improve chromatographic performance [58, 60].

$$H = \mathrm{A} + \frac{B}{u_x} + Cu_x = 2\lambda d_p + \frac{2\gamma D_M}{u_x} + \frac{f(\mathrm{K}){d_p}^2 u_x}{D_M} \qquad \textbf{(1.1)}$$

Where u_x symbolizes the linear flow rate or velocity, λ the structure factor of the column packing material, γ is a constant termed tortuosity or obstruction factor, K is the retention factor for an analyte and D_M is the analyte diffusion coefficient [58].

A, B and C are constants and respectively relate to multiple flow paths, longitudinal diffusion and finite equilibration between phases. The first term arise because molecules of the same analyte can elute at different times, as they can flow through different paths along the column. This results in band broadening and reduced efficiency. The employment of particles with low diameter can circumvent this problem, as it allows a more uniform flow through the column, thereby reducing the multiple path term (A) of **equation (1.1)** [61].

B or longitudinal diffusion related to the analyte diffusion along the axis of the column, from areas of high concentration to areas where it is lower. Thus the increasing of u_x will result in the decrease of B, as the analytes will spend less time in the column, reducing this term of the equation [61].

Finite equilibration between phases, simply called mass transfer (C), corresponds to the analyte equilibrium between the mobile and stationary phases. Small particle diameter will reduce the band broadening which exists in high performance liquid chromatography (HPLC) owing to the fact that analyte diffusion is not homogeneous. This occurs in certain larger particle size columns and lower pressure providing instruments, as some analyte molecules are retained in the stationary phase, whereas others continue moving forward, resulting in an unwanted larger value of C [61].

Concluding, by employing particle with lower d_p, will directly reduce the constants A and C from **equation (1.1)**. Additionally, it will indirectly reduce the constant B as the instrument is technologically capable of providing high pressure to the chromatographic column [61].

Altogether, this will result in lower values for H (also known as HEPT), which leads to a higher efficiency [58, 60, 61].

As illustrated in **Figure 1.15**, the efficiency of columns with smaller particles changes less dramatically, when the flow rate is increased. This enables a reduction in analysis time and enhances efficiency in UPLC, when compared to HPLC [58].

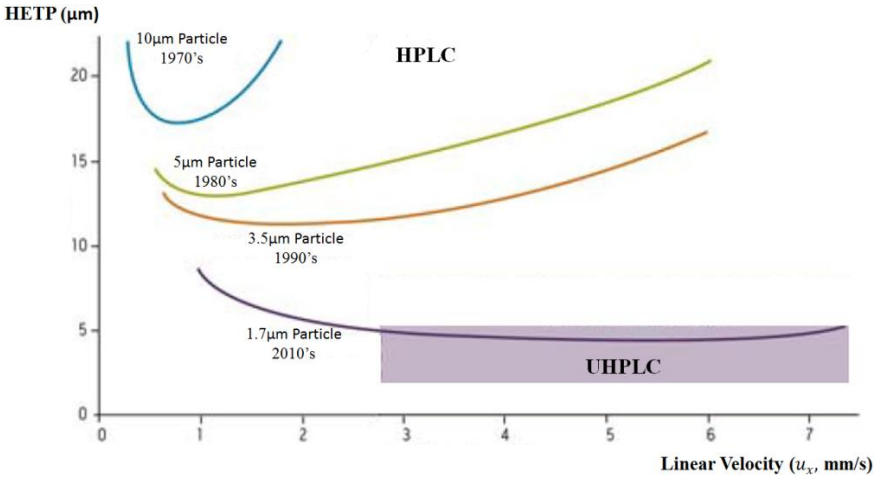

Figure 1.15 *Variation of efficiency (HEPT) in function of* u_x [60].

As provided by the illustration, columns with particles with diameters of 1.7 µm provide a higher efficiency than any other used in HPLC systems.

The highest chromatographic efficiency for UPLC systems is within the mobile phase and analytes flow rate velocity interval comprehended between 3 to 7.5 mm/s [60].

Contextualizing with this project, UPLC introduced in 2004 by Waters under the trademark UPLC™, offers enhanced chromatographic resolution and more intense peaks. This reduces the risk of peak co-elution and provides a reduced analysis time [62]. This minimizes one of the main constrains that influences accurate mass acquisition by a TOF

analyzer: the minimum number of points, i.e. minimum acquisition width which defines a peak [63]. Summarizing, narrower chromatographic peaks contribute for a more reliable and accurate mass (AM) detection.

1.4.3 Electrospray Ionization

In the context of LC-MS, within the ionization chamber (**Figure 1.16**) which is at atmospheric pressure, the LC eluent containing analyte and solvent molecules is introduced in the source, usually at the same flow rate of that selected in the LC run [64]. The typical ESI flow rate for toxicological analysis ranges from 0.1 to 0.6 mL/min [6, 8, 9, 59, 65, 66].

The eluent passes through the capillary/needle that has a high potential difference applied to it, opposite to that of the counter electrode and within the range of 2000 to 5000 V. The voltage applied in the needle will define either if the eluting analyte and solvent molecules will be positive or negatively ionized [64, 67].

The liquid protrudes from the ESI capillary metallic tip in a cone shaped fashion denominated “Taylor cone”, which has an excess of “molecular ions” with the pre-selected charge sign. The uninterrupted flow of charge from the metallic contact to the sample solution occurs via an electrochemical reaction at the surface of the needle. In positive ion ESI the dominant reaction is oxidation, whereas in negative ion ESI it is reduction [64].

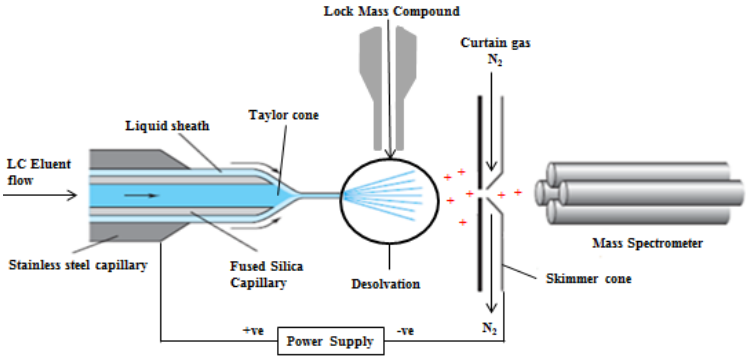

Figure 1.16 *Overall illustration of an electrospray ionization chamber in positive mode.*

In order to ensure analytical accuracy and reproducibility, a lock spray needle is present in the ion source, from where a lock mass compound is sprayed at the same time analyte and solvent molecules enter the chamber. Lock mass compounds are of known concentration and mass and are injected at a flow rate several times smaller than of that applied in LC and in the ESI capillary [9].

Currently, modern apparatus have being developed and successfully applied in order to avoid the drawback that the lock mass approach can originate, like ion suppression, potential mass interferences and solvent gradient effects [68].

Due to the electric field gradient between electrodes and the electrostatic repulsions between the charged molecules within the Taylor cone, its surface reaches a tension limit, designated *"Rayleigh"* limit (**Figure 1.17**). At this point Coulombic repulsion of the surface charge will be equal to the surface tension of the solution. This event will force the charged particles to exit the cone. agglomerated in the form of droplets. The difference of potential within the ESI source will make the droplets traverse the space between the two electrodes [61, 64].

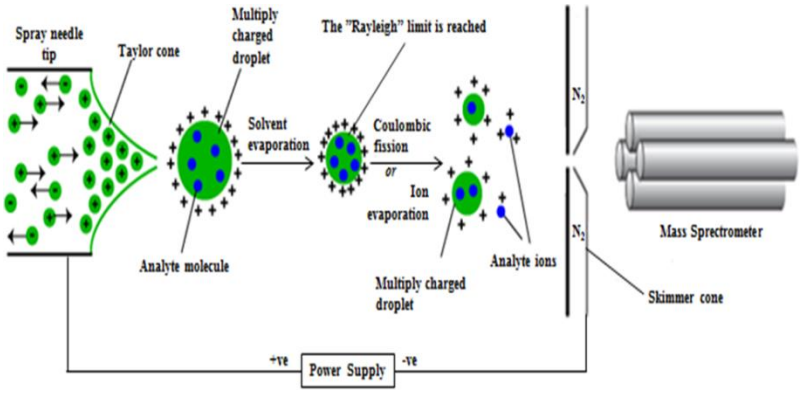

Figure 1.17 *Schematic of the mechanism of ionization in positive ion mode.*

The desolvation process and ultimately the formation of free analyte ions can be explained by two different but equally accepted mechanisms: Coulomb fission or ion evaporation [64].

Similarly to what takes place in the "Taylor cone" the Coulomb fission explanation assumes that, along the chamber's atmosphere, the increased charge density due to solvent evaporation causes droplets to divide gradually into smaller ones, which eventually will only consist of single ions with the intended charge. The second mechanism supposes that the increased charge density that also results from solvent evaporation will originate Coulombic repulsions to overwhelm the tension in the liquid's surface. This will ultimately release gas phase ions from droplets' surface [61, 64].

The ion source does not only produce condensed and ultimately gas-phased charged analytes ($[M+H]^+$ or $[M-H]^-$), but also neutral species and clusters of ions containing neutrals. To achieve optimal desolvation, separation of the interferences and the analytes, and to introduce them into the vacuum at the MS entrance, two flows of neutral gas are continuously introduced at both ends of the ionization chamber: the sheath gas and the "curtain" gas [61, 64].

The first consists in a flow of neutral specie in the gas phase that round the needle tip. Strategically off-axis positioned, it contributes to the separation of ions from neutrals in the outer region of the spray, generally constituted by smaller, lighter and more desolvated droplet. This way, additionally to its role in the desolvation *per se*, the off-axis positioning maximizes the amount of desolvated analyte that enters the mass spectrometer while selecting against the unevaporated droplets [61, 64].

At the other opposite of the capillary the "curtain" gas flows between both sides of the MS entrance. Normally composed by molecular nitrogen, it "screens" the ionized species at atmospheric pressure into the MS vacuum. Furthermore, it drives neutral species away from

the MS orifice, and allow the entrance of charged species, as they are impelled by the stronger electrostatic effect induced by the referred electric field gradient [64].

Generally, in electrospray little fragmentation of analyte occurs and mass spectra are simple. However, fragmentation by ESI can be intentionally increased by collisionally activated dissociation, achieved by adjusting the voltage at the counter-electrode cone. This enables the introduction of fragments into the MS vacuum for mass analysis, which can be useful, for example, in determining the identification of an analyte. However, the resulting product ion spectra are known to be of complicated interpretation [61].

The ESI ionization mode duality in toxicological analysis is greatly inclined for ESI positive mode, as most of known toxicologically relevant compounds have basic properties. The negative mode is mostly used for diuretics, barbiturates, some organophosphates and pesticide [69].

1.4.4 StepWave™

The analyzer employed in this thesis encloses a feature that maximizes sensitivity and robustness of the analytical process. The StepWave™ assembling is based in the stacked ring ion guide technology. **Figure 1.18** (Top) depicts an internal longitudinal plan of the off-axis design of this part of the instrument [70].

The device maximizes ion transmission from the ion source to the first quadrupole (Q) by exhausting neutral contaminants, excess solvent and high gas flow, illustrated by the red pointed arrows in the illustration [70].

This filtering step maximizes sensitivity by increasing the passage exclusivity for analyte charged molecules (blue pointed arrows) to the mass analyzer, resulting in an enhancement of the overall signal to noise ratio (S/N) up to 25 times [70].

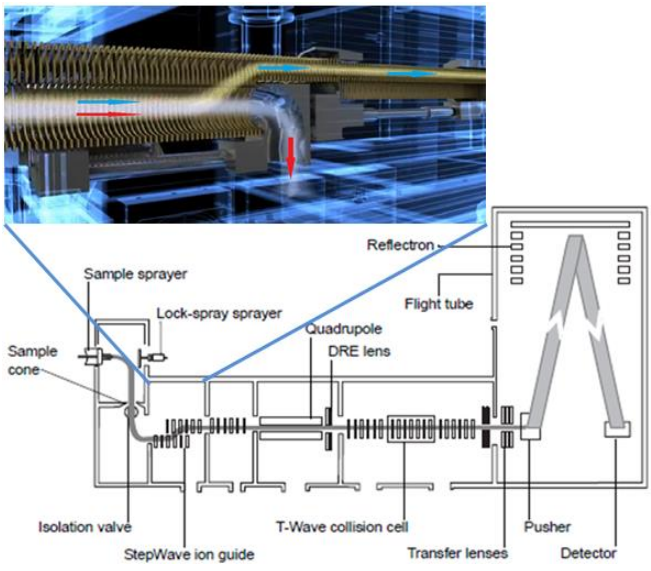

Figure 1.18 *UPLC-ESI-TOF/MS schematics with an in focus of a longitudinal cut view of the Off-Axis StepWave™ [70].*

The analyzer robustness is also increased as the filtration for instrument hazardous contaminants “sterilizes” the upper ion guide (blue arrows), which increases the longevity of the devices that constitute the mass spectrometer and detector [70].

1.4.5 Time-of-Flight and High Resolution Mass Spectrometry

In contrast to tandem MS instruments, TOF/MS instruments make possible a comprehensive registration of data. Targeted as well as non-targeted search procedures for drug screening can be performed on the same set of data [71].

TOF analyses involve accelerating a group of ions, in a brief burst, towards a detector. **Figure 1.19** illustrates, in this experimental context, what is expected to happen within the analyzer.

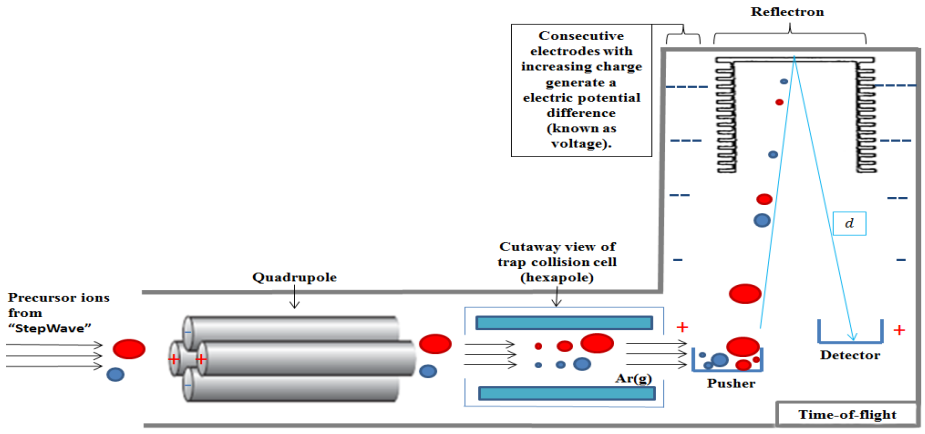

Figure 1.19 *TOF/MS analyzer and the route taken by ions inside the Xevo G2-S QTof™.*

After a previous filtering by the StepWave™, the positively charged molecules originated in the source (ESI) enter the Q where each ion's potential simply accelerates, as optionally, the capability to filter specific ions was not adopted in this experiment [60].

Then, in the collision cell two types of fragmentation energies will be applied upon the entering analytes (subject covered in **section 1.4.6**). Finally, after passage through the hexapole the ions enter the TOF *per se*. There the charged particles will be fired into a vacuum by a pusher. This stage is not so linear and can raise resolution problems which will be addressed later in this section [60].

In the tube, because all charged ions are subjected to the same electrical field, those with lower masses evidence grater velocity and a lesser time interval travelling a stipulated "flying" distance before striking the detector (see, **equations (1.2)** to **(1.7)**) [60].

The represented equation system, mathematically elucidates the role of mass (m), time (t) and distance (d) in the detection.

Equation (1.2) plots the kinetic energy (1/2 mv^2, where v is the velocity) applied by the pusher with an "equal" intensity to all particles, at a certain momentum or "push".

$$\frac{1}{2}mv^2 = qU \qquad \textbf{(1.2)}$$

In theory this kinetic energy is equal to the potential energy (qU) of each specimen, being q the individual charge of a particle and U the electrical potential difference (or voltage) that should be the same at the pusher stage. However this is not strictly observable, which may raise problems in resolution (discussed later).

Equation (1.2) can be rewrote in the form of **equation (1.3)** that depicts the equation in order to v.

$$\Leftrightarrow v = \sqrt{\frac{2qU}{m}} \quad \textbf{(1.3)}$$

Given that v is equal to the distance travelled over time (d/t), further modification of the equation to m is described in **equations (1.4)** and **(1.5)**.

$$\sqrt{\frac{2qU}{m}} = \frac{d}{t} \quad \textbf{(1.4)}$$

$$\Leftrightarrow m = \frac{2qU}{d^2}t^2 \quad \textbf{(1.5)}$$

In **equation (1.5)** the term $\frac{2qU}{d^2}$ may be grouped as an unit-less constant if the molecule is in a neutral environment in terms of charge. However, in a mass spectrometer, molecules are ionized (in the ion source) and there is a charge gradient along the analyzer, turning q into a variable. Hence, at **equation (1.6)** exclusively U and d are grouped so they can be simplified to the unitless constant k.

$$\Leftrightarrow \frac{m}{q} = kt^2 \quad \textbf{(1.6)}$$

As for that, in **equation (1.7)** it can be stated that the mass with an attributed charge varies with the square of time.

$$\frac{m}{q} \propto t^2 \quad \textbf{(1.7)}$$

So if a certain charged particle has a relative large mass in comparison to others, in a mixture, it will take more time for it to travel d.

Concluding, the segregation and separation of the mixture of charged particles are made in terms of mass-to-charge ratio (m/z). The change form q to z marks the transition from coulombic to the dimensionless quantity formed by dividing the mass number of the ion by its charge number, which is used for data presentation in a mass spectrum.

However, because in the source (ESI) the voltage is not evenly distributed along its area where ionization occurs, as illustrated in **Figure 1.20**, there will be occasions where particles of the same species, sharing the same exact mass, will travel in the vacuum at different velocities. This contradicts what was represented in the previous set of equations, which states that to particles with same mass, it will be attributed a similar kinetic energy [72].

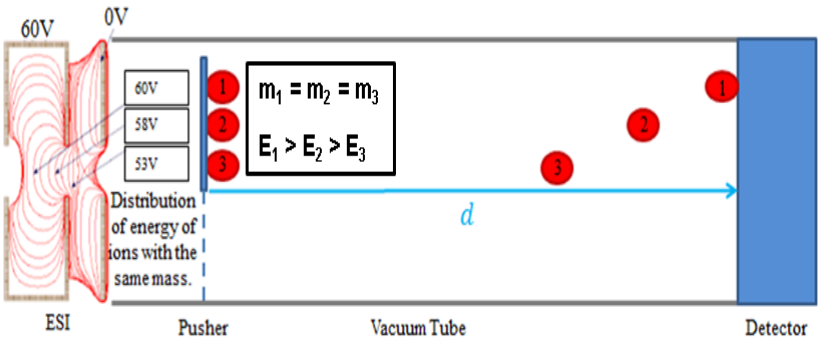

Figure 1.20 *Schematic of a linear TOF and flight of 3 ions with equal mass* [72].

As illustrated by **Figure 1.20**, there will be peak multiplicity in the spectrogram as molecules with same m/z will be detected at different times. This will influence the resolving power (RP) of an instrument [72].

Certain precaution can be adopted to avoid this effect, such as application of a pulse with the lowest duration possible (in the order of the nanosecond) or to provide a source of ions that provides the nearest to uniform equipotential conditions [72].

All the referred features introduced by a reflectron contribute to the exponential increase of the resolution and the RP [72].

RP is the ability of a mass spectrometer to separate ions with different m/z values measured having in reference the full width half maximum (FWHM). The latter, expressed in **equation (1.8)** is applied to a wide variety of MS analyzers including the TOF/MS [5].

$$\mathrm{R_{FWHM}} = \frac{\mathrm{m/z}}{\Delta \mathrm{m/z}} = \frac{t}{2\Delta t} \qquad \textbf{(1.8)}$$

Where t is the flight time of the ion and $\Delta \mathrm{m/z}$ and Δt are the peaks widths at 50 % level on the mass and time scales [73].

Another option is the introduction of a reflectron (see **Figure 1.19**) which functions as an electrostatic mirror to compensate the disparity of the kinetic energy. This will not equilibrate the energy at the source, but will decrease the flight distance for ions with less energy at the pulse and the reverse for the others with higher energies, as illustrated in **Figure 1.21**. Simultaneously, it will comprehensively decelerate ions with higher kinetic energy, until the velocities of the homologous ions reach equal intensity [72].

An apparatus with a reflectron, as that used in this work, also increases (doubles) the flight distance which, alone, increases the resolution of chromatograms and spectra [65].

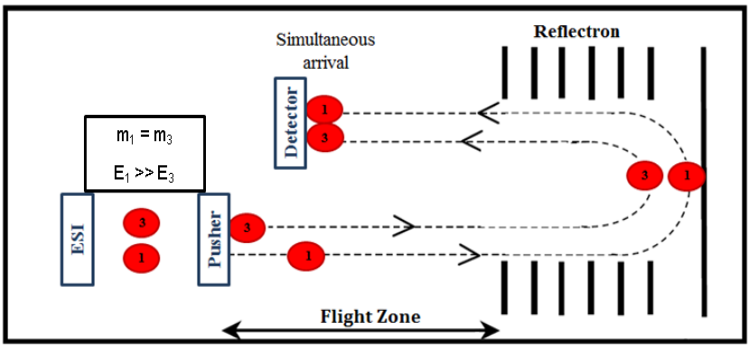

Figure 1.21 *Schematic of a TOF equipped with a Reflectron and the principles that are applied with it: Acceleration, Reflection and Deceleration* [72].

High MS resolving power is necessary to separate adjacent peaks from one another and to ensure that only one kind of ion contributes to the measurement. Several key factors must be optimized and considered to achieve high mass accuracy with good precision. These factors include peak shape, ion abundance, the referred RP, and calibration [73].

Resolution of a TOF is typically comprehended between 15000 to 26000 ($\frac{m/z}{\Delta m/z}$). Hence the problem raised by interfering peaks of ions with the same nominal mass, but different exact masses of intended analytes, may be solved partially or completely. It also allows the determination of the charge state of multiple charged ions from their isotopic mass spacing in isotope pattern studies. Additionally the S/N is improved owing to the grouping of ions into narrower peaks, which increases the peak height.

In sum, the greater the resolution the better, if it does not interfere with sensitivity [73, 74]. Additionally TOF detectors are the cheapest high-resolution mass spectrometers (HR(MS)), with consistent unique features in terms of acquisition speed and m/z range [74].

All the above characteristics provide TOF the technology to produce a full-scan, independent of set masses, with a molecular weight (MW) detection capability to the nearest 0.001 Da (dalton), compared with 1 Da in conventional MS [65].

Theoretically, this LC-HR(MS) has the requirements for a sensitive screening of (polar) targeted compounds and/or their metabolites, and to discover non-targeted compounds that can be of toxicological relevance in complex mixtures.

1.4.6 Data-Independent Acquisition

Unbiased data-independent acquisition (DIA) strategies attempt to overcome the disadvantages of its counterpart, data-dependent acquisition (DDA), in what concerns STA screening [75]. Differences between these two techniques will be discussed in **section 1.6**.

In summary, DDA consists in a pre-selection of a nominal or exact m/z, provided by a MS scan cycle, and then through an additional multiple reaction monitoring (MRM) cycle it is acquired the intended fragmentation spectrum.[62,69] This approach raises limitations such as irreproducibility of precursor ion selection, underdamping and long instrument cycle times [75].

Whereas for DIA experiments, two almost simultaneous and intercalated acquisition functions with different collision energies are configured in the collision cell. The low energy function (LE) and the high energy (HE) functions with a collision ramp energy, are depicted in **Figure 1.22** [76].

The analyzer/detector illustration describes a parent ion scanning, being the energy in the collision cell low enough not to fragment the charged particles (blue and red circles). The lower illustration describes the ensuing HE function. In this case, the smaller circles illustrate the respective product ions and their relative "flying" velocities towards the detector, when compared to their parent ions.

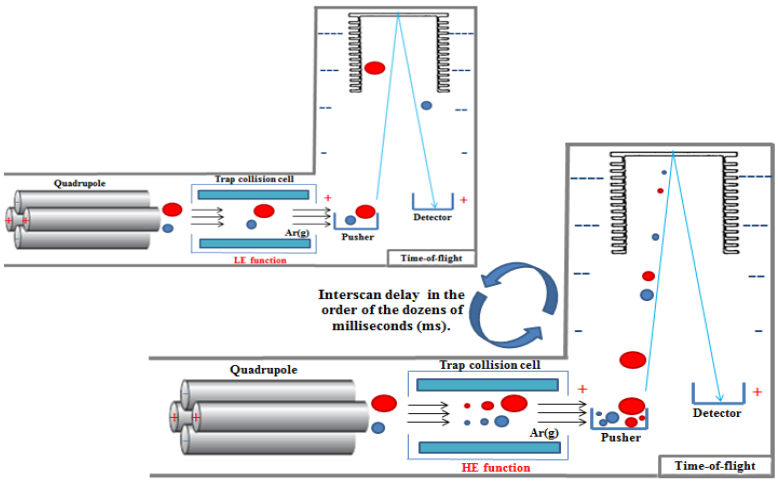

Figure 1.22 *Route of charged particles in a (HR)TOF/MS analyzer with DIA.*

In the detector, data being collected will be then separated into two data channels, with the instrument spending not more than 0.1 s on data acquisition for each channel[77]. Hence two alternating MS spectrums (**I** and **II**) are algorithmically displayed (*see* **Figure 1.23**) providing AM, intended isotopes, adducts and, most relevantly, fragmentation information of a targeted compound or of an unknown [6].

In the case of usage of a quadrupole TOF analyzer (qTOF/MS) instrument, as it was the case in this thesis, because no qualifier parent ion is selected in DIA, the first quadrupole is set to static mode.

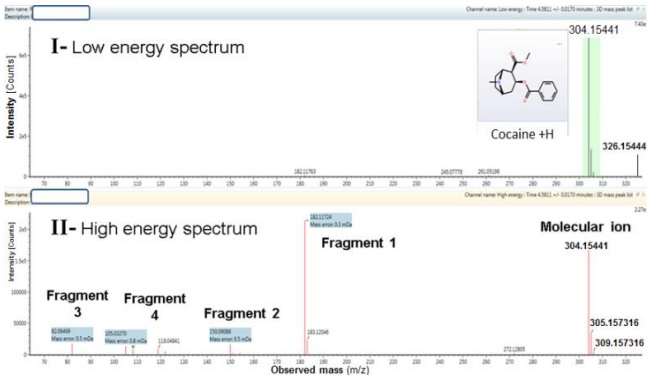

Figure 1.23 *Cocaine DIA spectra of a real forensic case, displayed in UNIFI1.7™ software.*

As it is observable, the data treatment software employed in this project enabled compound specific DIA results. The upper spectrum is relative to the LE data channel result, providing the exact molecular mass of the parent ion (304.15441 m/z), isotopes (305.157316 m/z and 309.157316 m/z) and sodium adduct of cocaine (326.15444 m/z).

The lower spectrum depicts the HE spectrum which provides fragments exclusively of cocaine, algorithmically placed to the left of the parent ion peak. In this spectrum cocaine´s peak has decreased in detector counts when comparing to those of the LE spectrum, which

evidences loss of precursor ions for this compound, as a substantial amount was fragmented into product ions (fragments 1 to 4 in **Figure 1.23** (II)).

1.4.7 *UNIFI1.7*™

UNIFI1.7™ is a software tool from Waters Corporation™. This version (beta) is exclusively compatible to Windows 7™, 64-bit operating system (OS) and is still in development by the manufacturer. Hereby it is presented some of its more innovational features in the context of STA [78].

The software controls instrument systems and devices, and performs all data acquisition, analysis, processing, reporting, and other information management functions [78].

It allows the study of old samples as a database is maintained in a server and stores raw data and content items including results, sample identifiers, methods, and reports in a relational database management system [78].

It directly influences instrument robustness, contributing to its maintenance through a console of functions. With this application the operator can configure the instrument settings, monitor performance, perform diagnostic tests and maintain the instrument system and its modules [78].

This tool provides the possibility to create individual methods for instrument control and lists them for future use (see **Figure 1.24**) [78].

The "analysis method" acts as a shortcut in which it is stored all trial conditions, instrument control parameters, and data processing parameters. Additionally, a series of methods can be stored for future application, depending on the context of the analysis [78].

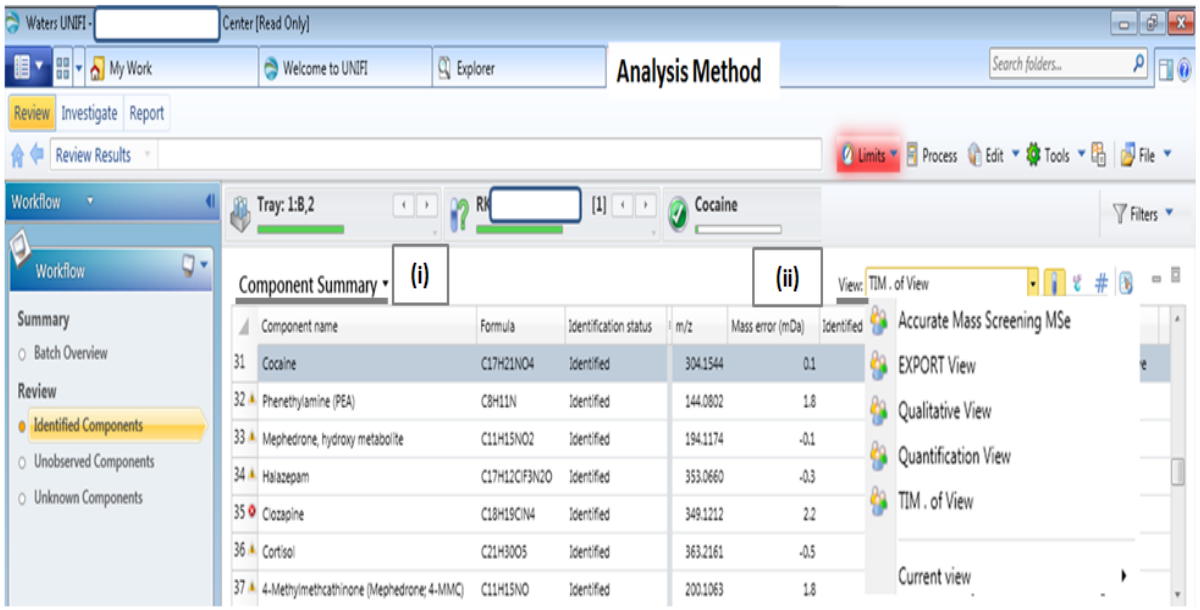

Figure 1.24 *"Analysis method" window of UNIFI1.7™.*

A list of identified compounds, indicated by **(i)** and denominated "Component Summary" is displayed with relevant information such as molecular formula, retention time (RT), status of identification (analyte presence in library or not), the exact mass of the protonated molecule and the mass deviation of the acquired spectrographic peak, relatively to the stored information in the library. There are other criteria possible to be displayed on the list **(ii)**, which is dependent in the selected "view".

"Analysis methods" specifies the following features [78]:

- The type of analytical experiment it is wanted to run;
- The compounds in one or more samples that are wanted to analyze. For example, in the case of the "exclusion" of compounds from the targeted library in the method specificity studies. The compounds to be "hidden" are those present in the "excluded" library (discussed in **section 3.1.4**);
- The instruments to use for data acquisition;
- The instrument control settings and parameters;
- The parameters that define how to process the data for analysis. This application defines the filter criteria to be applied in the data process. Again, this is of relevance for specificity studies, highlighted in **section 3.1.5**[78].

When reviewing old data, the software is able to assign different "views", which are dependent on operator's preferences for data interpretation (see **Figure 1.24 (ii)**) [78].

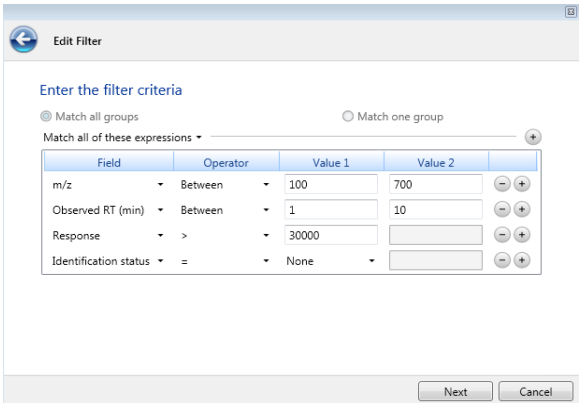

Figure 1.25 *UNIFI1.7™ "Edit filter" box.*

Among the already referred and other capabilities, the software is able to assign filters with a list of criterions possible to be inter-correlated, in order to provide a more specific list of compounds (see **Figure 1.25**). Hence, it is possible to attain a narrower list of chemicals which chromatograms and spectrograms are to be assessed, without compromising the sensitivity of the analysis.

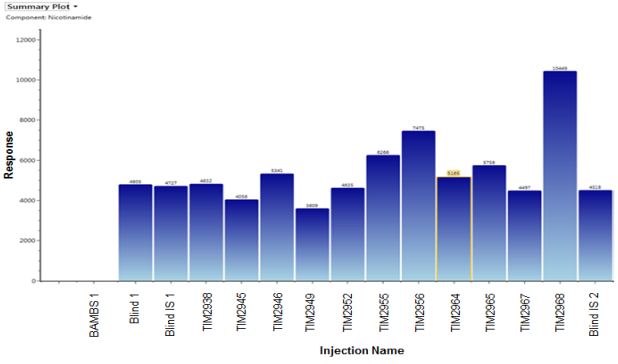

Figure 1.26 *UNIFI1.7™ "Summary Plot".*

Another property of this software is the ability of providing a compound specific qualitative and quantitative (in terms of response/detector count) comparison tool, designated "summary plot".

In **Figure 1.26**, it is illustrated an example of an endogenous substance (nicotinamide) present in the library of

compounds adopted in this thesis. From the plot it is possible to conclude that the referred substance is in every sample. Each column represents the nicotinamide response (for each injection). This catalogs the compound as a non-pharmaco-toxicologically relevant compound and promotes subsequent listing in the "excluded" library (see **section 3.1.4**).

Another feature to be presented is the capability of this software to import molecular structures as a *.mol* file. This format is directly downloadable from *ChemSpider*^{TM} or can be provided by any chemical drawing software.

This particularity is of high importance in the development of the semi-targeted screening method, as the software has the ability to attribute molecular structure to product ions, which are displayed in the HE spectra of an unknown not present in the library of compounds. In **Figure 1.27** it is given the example of N-dealkyl-hydroxyquetiapine, a non-targeted metabolite of quetiapine, identified with resource to the highlighted feature.

In the image, **(a)** is correspondent to the low collision energy spectrum and **(b)** corresponds to the high collision energy spectrum.

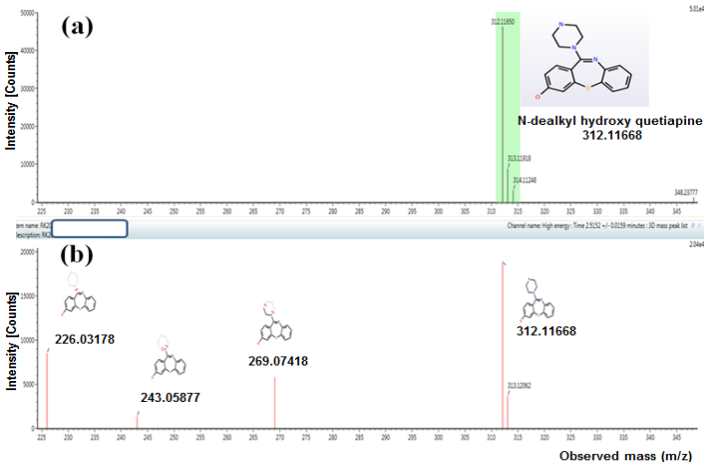

Figure 1.27 *N-dealkyl-hydroxyquetiapine molecular (above) and product (below) ion structures.*

Another important property is the ability of displaying chromatographic peaks of product ions that are common to different compounds present in the same sample. **Figure 1.28**, illustrates a real forensic case where cocaine was identified. The chromatographic information reveals that there are 4 fragments of cocaine that are common to other chemicals present in the sample.

Graphic **I** corresponds to the total ion chromatogram (TIC) in which all the detected peaks are displayed, irrespectively of being present in the database of compounds, or absent, being accounted as unknown small molecules. Scheme **II** is the extracted ion chromatogram (XIC) for every component with m/z equal to 304.1544.

Graphics **III** to **VI** are the XICs of fragments of the pharmaco-toxicologically relevant compound present in **II** (cocaine in this case). Respectively, they correspond to the product ions with 182.1172, 82.0647, 105.0327, 150.0909 m/z.

In **Figure 1.28** the fragments present in **IV**, **V**, **VI** are both product ions of cocaine and benzoylecgonine, which eluted at the minute 2.98.

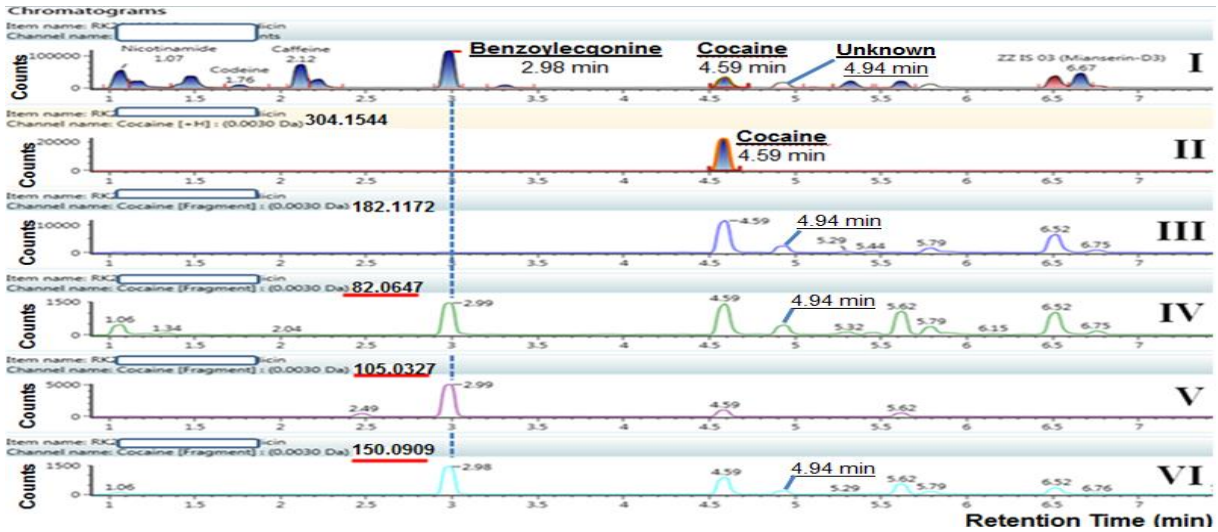

Figure 1.28 *UNIFI1.7™ common product ion appointment tool.*

Normally the non-targeted peaks (*eg.* substance eluting at 4.94 min in **Figure 1.28**) correspond to non-listed metabolites of the compound being analyzed. However, there is always the probability of those complete unknown peaks to correspond to PTRCs which identification is of utmost importance for the forensic report.

This is the problematic that fueled the need for the assembling of the semi and non-targeted screening methods, developed in this project.

1.4.8 Targeted Screening

Due to several hundreds of thousands of intoxications *per* year with drugs of abuse and pharmaceuticals, fast screening methods are a necessity for the determination of these xenobiotics in forensic intoxication cases [79].

In the last 10 year, the development of HR/MS instruments allow the provision of high mass accuracy and resolution in full scan mode, enabling AM screening of a theoretically unlimited number of polar organic pharmaco-toxicologically relevant compound [79, 80].

This section describes the first of the three screening methods introduced by Krauss *et al*. [81]: target, semi-targeted and non-targeted screenings [81].

The classical automated targeted analysis approach uses pure standards to serve as reference while the MS full scan is taking place. Those reference standards provide the RT information of the intended compounds, which is stored in a database (named targeted library for simplification). These libraries are normally in-house customized but the bulk is often commercially acquired. In addition to RT they might store information of the exact mass, fragmentation from tandem mass spectrometry (MS/MS), molecular structure, and other information [82].

A targeted library can contain up to several hundred substances [83], which make it a resourceful tool for practical screening of PTRCs where reference standards are commercially available [82].

Indeed, Gregov [84], resorting to a conjugation of results from distinct STA methods (GC, GC/MS, HPLC-MS/MS) reported that, between 2000 and 2003 only 80 common drugs, to which respective pure standards were available, covered 87% of all findings in the forensic laboratory where the experiment took place [84].

The utility of these databases is mainly justified because of the knowledge of the RTs obtained under the method´s specific chromatographic conditions [84].

However, if not obtained commercial source, the database assembling is very laborious and costly as all reference standards have to be purchased and the RT individually measured. Additionally, not always the substances to which RT measurement is required are easily provided by synthesis laboratories, as is the case of drug metabolites [84] and designer drugs.

1.4.9 Semi-Targeted Screening

As stated in the last section, HR/MS enable the differentiation of thousands of peaks form the background noise, which largely surpass the target compound library range. As for that, those peaks remain unidentified and might be correspondent to non-relevant chemicals, such as endogenous compounds, or they simply might be a result of analytical interferences. However they also might correspond to a PTRC hit [79, 80].

As for that, in a semi-targeted (also referred to as “suspect”) screening, it is attempted the identification of those non-identified but detectable peaks [82].

In this screening step a library of compounds called semi-targeted library is assembled with the ultimate objective of providing all the information that a target library would provide, except for compounds´ RTs. Hence it is called “semi” because even though all the compounds to screen for are known beforehand, it lacks the reference standard information [82].

In this screening method, with resource to specific software, chromatographic peaks with exact mass are correlated to the AMs present in the semi-targeted database. These AMs derive from imported molecular formulas and are compound specific (except in isomerism situations). Parent ion peak and suspect compound affinity is corroborated by the agreement between measured fragments´ exact mass and the suspect fragment´s AM stored in the database [82].

It has been reported (Kern *et al*. [85] and Gómez-Ramos *et al* [86]) that confirmation of suspect-library hits can be performed by further analysis of suspect´s retention behavior; by expert comparison of spectra acquired by multiple fragmentation energy mass spectrometry (MS^n) methods; and by knowledgeable judgment of spectra obtained in both ionization polarities [85, 86].

If such a screening strategy is feasible, it will alleviate the dilemma of requiring reference standards *a priori* and opens the door for the fast detection of compound classes for which reference standards are not easily accessible [82].

However, in the context of this thesis, the rule followed was that, for unambiguous confirmation of the presence of the suspects, it is always needed to calibrate the LC-MS system with an isolated pure standard of the compound of interest [82].

Additionally, the semi-targeted screening was performed as a post-targeted screening step, in which the results of the first will complement the positive hits obtained in the latter [81].

1.4.10 Non-Targeted screening

Traditional targeted approaches provide good sensitivity and reliable identification (and quantification) of the targeted compounds and have successfully been applied for several decades. However, database based screenings account for a major drawback, as all the compounds which are absent from the selected compound list will always be missed in the analysis [87].

Nowadays, HR(MS) and AM acquisition permit the automated detection of the “true” unknown compounds and further enable the deconvolution of the respective chromatogram peaks into spectra.

However the resulting raw total chromatogram will reveal several thousands of unknown peaks in a single whole blood sample [88].

In order to mine these chromatograms for useful information, powerful software tools became a necessity. Programs such as *UNIFI1.7™* provide the resources to filter down the “true” unknowns list to a feasible quantity for further analysis [89].

Most importantly, the program enables the examination of old full scan data files in a retrospective way, in order to look for pharmaco-toxicologically relevant candidate peaks in whole blood samples. Without this feature, non-targeted screening would not take place to start with [87].

For a question of semantics and to avoid confusions, unknowns being treated in non-targeted screening will be denominated “candidates”.

Hence, a candidate is a complete unknown chromatographic peak, to which molecular formula can be attributed from the derivatization of both obtained exact mass and isotope pattern (to be discussed in **section 3.3.2**) [90].

The next stage of the non-target screening corresponds to the elucidation of the chemical structure, as a large number exist for each calculated molecular formula [88]. Hence the procedure develops with the searching, in the Internet, for possible structure identities in general databases [89], such as *ChemSpider™*. To assist the structure characterization, the obtained high and low collision energy spectra are compared with in the Web databases containing MS/MS fragment ion spectra with AM [89], such as *mzCloud™*. However, the use of mass spectral libraries for confirmation of compounds is still limited for certain vendors of LC-HR(MS) (Thermo Fisher Scientific™ in the case of *mzCloud™*). Hence, at this stage, mass spectra libraries are small and the compatibility of spectra is limited between different instruments [91].

Reverse search has not been fully recognized and valued to this point [87]. The current study took full advantage of this possibility and attempted to evaluate the contribution of its application in a STA context. Additionally, this will be the ultimate tool for the search of the so called “designer drugs” discussed in **section 1.3.2**.

1.5 Additional theory for method evaluation

In this section definitions that were employed in the evaluation procedures will be clarified. These experiments had as main purpose the elaboration of an objective insight of the inherent quality of the GUS method developed in this project.

1.5.1 LOD, LOF and LTC

This project didn’t cover PTRC’s upper limits of both detection and identification. Subsequently, for disambiguation, “limit” throughout this thesis signify “lower limit”.

The definition of limit of detection (LOD) was fixed by the Conference Report II as “the lowest concentration of an analyte in a sample, in which the bioanalytical product can reliably be differentiated from background noise” [92]. Its value is always inferior to the lower limit of quantification (in studies where it is of relevance). The most common approach for LOD definition, in an experiment, is to set the detector to exclusively consider the peaks with heights (signal) equal, or greater than the amplitude between the highest and lowest point of the baseline (noise) [93].

In this work´s screening method, LODs of a selection of 198 PTRCs were determined and the results were correlated with each compound's respective lower therapeutic concentration (LTC). The latter is defined as the reported minimal concentration, in whole blood, in which a drug will manifest its therapeutic properties in the user [94].

Limit of identification (LOI) is the concentration at which chromatographic and spectral information meet enough criteria in order to be appointed as unambiguously preset in the sample. The criteria are subjective and are either defined by operator's toxicological knowledge and/or through application of software filtering tools [95].

In this thesis context, for an estimative 95% of the PTRCs accounted for in the developed overall screening, compound identification was confirmed through the analysis of fragmentation patterns. Hence, limit of fragmentation (LOF) was the main criterion adopted for peak identification. It is defined as the concentration limit at which a selected PTRC provides fragment(s) in the DIA's HE function spectrum.

To increase result robustness, at least 3 distinct but compositional homologous matrices should be analyzed and the mean of the resulting data calculated for LOD and LOF unambiguous determination [6].

1.5.2 UP, FP, FN, CP

For evaluation of the method´s targeted screening, four possible types of results were delineated:

1) *UNIFI1.7™* positive (UP): were results that, after instrument´s detection, were identified as positive hits by the operator.

2) False Positive (FP): which comprehended results that, regardless of detection by the instrument, were appointed as false hits. These chromatographic/MS peaks did not satisfy the identification criteria, such as peak shape, provision of fragment ions, satisfactory m/z and RT errors, presence of metabolites/ precursor compounds and presence of adduct(s).

3) False Negatives (FN): which corresponded to small molecules that were not detected by the method´s software or were classified as FP. However, these substances were identified and quantified by parallel methods and presented in the RKA´s laboratory information management system (LIMS).

4) Confirmed Positives (CP): UP results that were corroborated by parallel methods were designated as CP. This classification was implemented with regard to cross evaluation between results obtained from *UNIFI1.7™* and those from other instrumentation considered in the LIMS.

1.5.3 False positive frequency and sensitivity

The Instrument´s targeted screening capability was evaluated in terms of FP frequency and sensitivity. Additionally, validation of software filters was performed.

FP frequency, expressed in **equation 1.9,** is presented as the quotient between the total of instrument detections classified as FPs and the sum of all the hits [96].

$$\text{FP frequency} = \frac{\text{FP}}{(\text{FP+UP})} \quad \textbf{(1.9)}$$

Roman *et al* [71] stipulate that in a targeted screening employing a library of compounds comprising 240 drugs, it was acceptable a FP frequency of 5% [71].

Sensitivity, expressed in **equation 1.10**, is the ability of the method to correctly identify small molecules, without missing any PTRC [96]. It consists in the quotient between the number hits classified as positives (UPs) and the sum of UPs with the total PTRCs missed in the screening (FNs).

$$\text{Sensitivity} = \frac{\text{UP}}{(\text{UP+FN})} \quad \textbf{(1.10)}$$

Accordingly to Roman *et al* [71] it is expected that a screening method in a STA should provide a sensitivity of 100% [71].

1.6 Literature review

To better comprehend LC-MS based broad screening procedures, able of covering a wide range of toxicological relevant compounds, hereby are presented two independently related dualities: STA based on product ion spectra *vs* GUS based in high resolution accurate mass detection (HR/AM); and DDA *vs* DIA [69].

For the first two different general screening approaches, the one based on product ion involves the usage of triple quadrupole, linear ion trap (LIT) (illustrated in **Figure 1.29**) and hybrid mass spectrometers (quadrupole ion trap or QIT). Any of them can provide information rich fragment ion spectra, to which can be applied search against libraries of reference product ion (fragment) spectra. These references are previously recorded on the actual or on a similar type of instrument, as there are reproducibility limitations in what concerns spectra results between LC-MS apparatus [4, 5, 7, 8, 83, 98].

The other GUS approach is based on HR/AM and it is performed either by TOF mass spectrometers (see **section 1.4.5**) or by orbitrap instruments [99]. In this case the compounds are identified by comparison of exact masses with accuracy of at least 4 decimal houses. Databases can be obtained commercially, recorded from available pure standards, or built based on theoretical data. The latter provides its main advantage which is the fact that accurate mass databases are reproducible as a PTRC´s molecular formula is [2, 3, 6, 73, 75-77, 99-106].

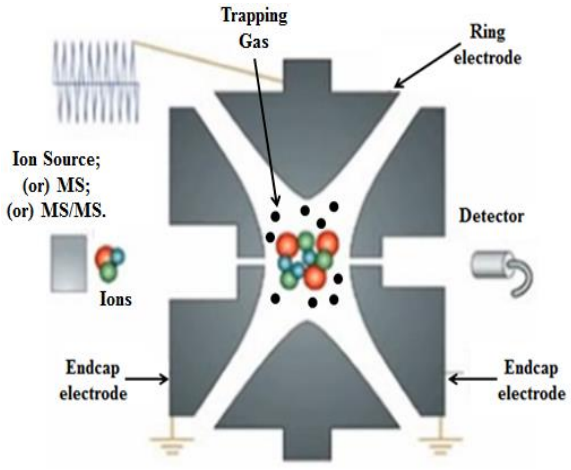

Figure 1.29 *Ion Trap Spectrometer [107].*

For instance, the instrument's software used in this thesis is able of calculating the exact mass and isotope pattern of intended compounds exclusively by importation of their molecular formulas. The reverse is also possible through usage of software tools that can probabilistically correlate an exact mass to hypothetical molecular formulas, while accounting information from isotope and fragmentation pattern. This is important for the identification of drug candidates in the chromatogram and is discussed in **section 3.3.2**.

Despite not being used in this thesis, the ion trap apparatus presented in **Figure 1.29**, is described in literature as somehow a screening counterpart to the method developed in this study, so its operating description is hereby briefly described. Although being capable of providing broad fragmentation information, this instrument is exclusive for product ion spectra acquisition methodologies. Hence, it provides nominal and not exact m/z values for parent and fragments ions displayed in its spectra [3, 107].

LIT process starts when charged particles, exited from a previous part of the instrumentation, enter the ion-trap which consists of a grouping of three electrodes: two endcaps electrodes and a central ring electrode to which variable voltage intensity is applied. The trapping stage commences when the 1st endcap electrode is charged by an alternate current (AC) and the polarity of the current is what will determine the entrance of the ions into the ion trap. However, their flux is conditioned mainly by the detector data acquisition and processing capability. Afterwards, the trapping gas molecules will stabilize the ions accordingly to a m/z determined by the voltage applied in the central ring electrode. After the ion trapping stage is completed, the 2nd endcap electrode initiates the detection stage by mediating the injection to the transducer, through a technique designed mass-selective ion injection. This consists in a sequential ejection of ions according to their mass by voltage increase in the ring electrode. This procedure is cyclical and it is what makes possible the MS^n scanning capability to every intended analytes trapped in the chamber [107].

Ion trap apparatus can provide either enhanced MS survey scan or (more importantly) enhanced product ion (EPI), or even both in the same analytical run, in the case of a hybrid triple quadrupole linear ion trap instrument (Qtrap). The main advantage provided by this instrument is its MS^n capability, which provides multiple product ion chromatograms for the same ion specie at different collision energies, increasing the "richness" of fragmentation information in the spectra [107].

The second duality is more software driven than instrument dependent. As an introduction, one of the main differences between DDA and DIA is the fact that the first is well established with both GUS based on product ion spectra and STA based on HR/AM acquisition, whereas DIA is rather limited to HR/AM acquisition based screening [2-4, 6, 76, 77, 100, 106, 108]. An exception is exemplified by Humbert, *et al*. [62], and discussed later in this review [62].

The favoring of HR/AM acquisition is explained by the deconvolution of chromatographic data into mass spectra in DIA (illustrated in **Figure 1.30**). It requires mass assignment to four decimal places, so the fragment ion spectrum may be correctly correlated with the parent ion spectrum. This way, accurate mass acquisition is convenient to differentiate between isobaric compounds of the same nominal, but differing exact masses [5, 8, 73, 83, 98, 101, 102].

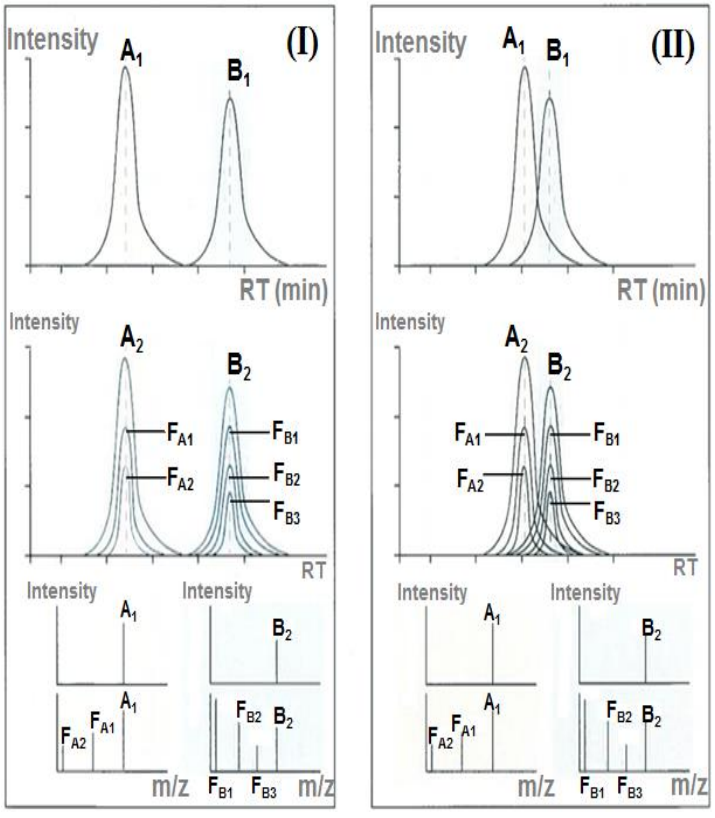

Figure 1.30 *Deconvolution of chromatographic data into mass spectra in DIA.* ***(I)*** *Example of peaks with distinct RTs.* ***(II)*** *Example of co-eluting peaks with a correct spectra alignment (homologous to those of* ***(I)****) possible due to specific deconvolution algorithms.*

The DIA feature of the software used in this thesis permited that, even when chromatographic peaks co-elute (see **Figure 1.30 (II)**), the deconvolution algorithms was able to align the parent ion in the low energy spectra (e.g. A_1 and B_1 in the upper spectrum) with the homologous ions present (now with less intensity) in the high energy spectra (e.g. A_2 and B_2 in the lower spectrum). More importantly, it allowed the correct allocation of the product ions (e.g. F_{A1} and F_{A2} for A_1) in the high energy spectrogram.

In a different way, DDA functions as an artificial intelligence-based on parent ion scan mode providing automatic and real-time

MS to MS/MS switching. This way, as in the case of DIA, there will be (at least) two spectra, one resulting from a low collision energy revealing the parent ion that will then activate a comprehensive MRM function in order to provide the respective product ion spectrum [75, 83]. The main difference between this approach and DIA is the fact that DDA defines the interval or the intended m/z it is wanted to analyse, whereas DIA provides an option of analysis that do not require any kind of narrow criteria prior fragmentation. Ultimately, this DIA capability is what makes it such a powerful tool for the tentatively identification of non-targeted compounds, and the "power" of this tool was assessed in the non-targeted screening (**section 3.3** of this work) [5, 8, 73, 83, 98, 101, 102].

Although very popular, there are some inherent limitations to the DDA approach, such as irreproducible precursor ion selection, undersampling and long instrument cycle times. For this reason, unbiased DIA strategies try to overcome those limitations [75].

As the subject is now finally presented, some examples will be shown to provide information about the screening methods and compare those in literature with the methodology developed in this thesis.

The literature study covers screenings techniques from a LC/ESI-MS method with intelligent data acquisition [7], to a state of the art UPLC-TOF-MS employing a STA screening HR/AM acquisition with DDA [6, 109].

The paper of Lafaye, *et al*. [7] describes a methodology not fully contextualized with a GUS procedure, as it does not describe a method to screen for drugs in a forensic or clinical context. However, as the study was in a metabolomics context to screen rat urine for targeted metabolites, which presence wasn't reportedly expected by the researcher, this method proved to be a good starter to introduce the subject [7].

The group's study dealt with the usage of liquid chromatography coupled to electrospray ion trap mass spectrometry for analysis of rat urine metabolome. The objective of Lafaye's team was to study how QIT instruments are well adapted to a first step of metabolism profiling. In the meanwhile, they developed a DDA technique from information rich MS^n spectra (providing nominal mass of parent and product ions), made available by QIT for further structural characterization of biomolecules [7].

Despite the well-established assumption of ESI spectra complexity (when applied high collision energies), as several dozen of m/z ratios may be recorded in a single chromatographic peak, the instrument's ion source exclusively provided the low energy function spectra [7].

To counterbalance the hypothetical spectral complexity which would result from a high collision energy function of the ESI, the team adopted instead a data-dependent MS/MS acquisition using a QIT analyzer to differentiate, in real time, metabolite form

fragment data, which dramatically simplified the database search. An example of the identification procedure is presented in **Figure 1.31**, which describes the identification of a fragment ion (not a phase II metabolite) of a phase I metabolite with a nominal mass of 385.0 m/z [7].

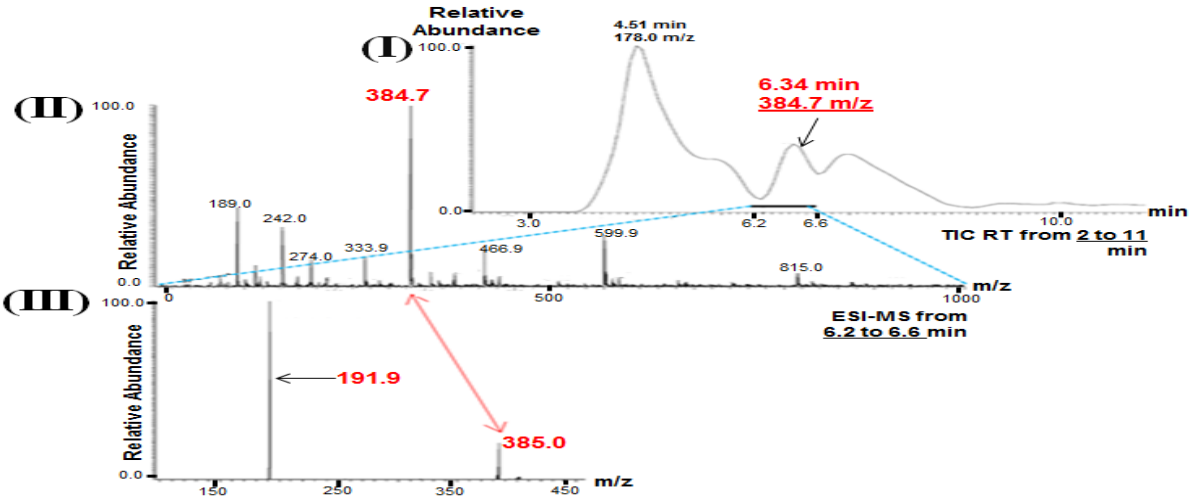

Figure 1.31 *Lafaye, et al. [7] DDA procedure.* ***(I)*** *Total Ion Chromatogram (TIC) with intended RT interval of interest highlighted;* ***(II)*** *corresponding deconvolution into ESI-MS low voltage spectrum (MS^1); and* ***(III)*** *MS^2 (QIT) product ion spectrum of the peak with 385 m/z of MS^1 spectrum. The dashed blue lines represent the deconvolution from chromatography to MS data [7].*

The previous figure exemplifies the worth of DDA mode, has it made possible the identification of the fragment with the mass ≈192 m/z, attained in the MS^2 spectrum displayed in **Figure 1.31 (III)**. However, it also evidenced some of the disadvantages of this intelligent data acquisition with product ion spectra (with nominal mass) as reference in the targeted screening. For instance, there is loss of RP when comparing Lafaye's method against a HR/AM based screening [2,6 7,109].

Another broad screening example is presented in the study of Dresens, *et al*. [8], which in 2009 reported on the identification of 700 drugs by multi-targeted screening with LC-MS/MS system and library searching. The library was in-house built based on electrospray ionization–MS/MS spectra and containing over 1250 spectrums, which included drugs of abuse, pharmaceuticals and toxic compounds of forensic and clinical relevance. Their approach comprehended a "scheduled" survey scan though 700 MRM cycles, DDA and usage of EPI spectra from a QIT scanning at three collision energies of 20, 30 and 50 eV [8].

As the library had spectral information about the product ion, for correct identification of the parent ion, this method exemplified a STA based on product ion spectra, in which it was used a DDA for data treatment facilitation [8].

The DDA software feature enabled a scheduled MRM (sMRM), which allowed the setting of time windows for transitions monitored in the survey scan. This is used to automatically select precursor ions as soon as they have been detected within the selected

time window where they were expected to be detected. All was done within the same analytical run [8].

Drensens research revolutionized through the employment of a MRM instead of using less resolved ESI or single MS scans which provide less efficient analyte detection. However, the method was not immune to sensitivity impairments [8].

In occasions the DDA mode was not activated even if the precursor ion was genuinely present in the sample and caught in the survey scan. In other occurrences, sensitivity was lowered because EPI spectra resolution was insufficient to unambiguous detect the parent ion from the survey, resulting in impairment of further sMRM fragmentation [8].

Another drawback was the limited number of spectra of metabolites in their library, which could be of contribution for the unambiguous identification of their parent drug [8].

Additionally, the protracting time-lapse of polarity switching, in many times raised the need of an additional run [8]. Nevertheless, comparing with the method developed in this thesis, the availability of "real time" polarity switching is an advantage, as this option wasn´t adopted in this projects´ screening method.

Finally and as well related to the analysis duration, the usage of MRM for parent ion scanning limited the number of compounds in their target library. Although 1250 spectrums is a considerable number, for further compound inclusion in the database, a longer chromatographic run should be adopted, which isn´t in accordance with the time efficiency goal of a STA.

Dularent, *et al*. [4] proposed an additional mean of employing a GUS based on product ion spectra in conjugation with DDA. This screening is different from the previously mentioned as it used a dual product ion library approach. The libraries together contained spectra directly related to 320 pesticides and metabolites. Dularent´s spectra libraries were generated by a LIT instrument. The MS^2 spectra library was generated using the base peak ions of the parent full-scan spectra library (MS^1), which contained the precursor ions. Therefore, the MS^2 product ion spectra library contained 450 fragment ion spectra of the 320 pesticides and metabolites (precursor ions), available as pure solutions [4].

Additionally, for the MS^3 spectra library, the precursor ions were those generated in the MS^2 scan. This resulted in an additional 430, second ion trap cycle, fragment ions acquired form the first 450 ion trap cycle (MS^2) fragment ions [4].

This approach's main strength resided in the availability of the well-developed 2nd IT cycle product ion library (MS^3), which functioned as an additional confirmation criterion for identification purposes. Supplementary, the capability of using data-dependent scanning and polarity switching after each cycle, in the same trial, proved to be very resourceful [4].

Another example of a STA based on product ion spectra but without resource to any artificially intelligent data acquisition is the work of Liu, *et al*. [98]. His paper highlights a new developed analytical feature that corresponded to a, at the time, novel fragmentation approach which consisted in providing voltage ramping and broadened mass window for activation (or fragmentation width) [98].

Their targeted library was based on the analytes' retention data and in "information-rich" MS/MS spectra of 780 standard drugs and toxic compounds. It was meant to screen for drugs in *postmortem* specimens (urine, serum and whole blood) using an ion trap mass spectrometer [98].

The "information rich" product ion spectrum contained fragments ranging from low to high m/z, provided by the higher voltage and lower voltage, respectively. The affinity between the product ion spectra empirically obtained and spectra in library was mediated by the NIST-based search algorithm. The latter also demonstrated high reproducibility between reported data from old cases and data gathered 6 months later from newer cases [98].

Their ramping voltage CID provided a significant number of product ions, which facilitated the characterization of analytes and lowered their limits of detection. As for that Liu, *et al*. [98] LC-MS/MS library search approach proved highly effective for broad preliminary screening and confirmatory analysis of drugs and toxic compounds [98].

In the context of GUS based on high resolution accurate mass detection (HR/AM), the paper in the literature with the largest compound library is that of Polettini, *et al*. [106]. The library was composed by approximately 50500 PTRCs (parent drugs and metabolites). The author took to the full extent the reproducibility of compound accurate molecular mass and its easy and free availability in million compound databases present in the internet [106].

Polettini's library is a subset of the Internet library "*PubChem™*" assembled by the National Center for Biotechnology Information (NCBI). Their approach attempted to overcome the main limitation of the usage of commercial libraries for the screening procedure in their capillary-electrophoresis (CE)-ESI-TOF/MS instrument, which was simply the fact that their limited compound content understates the RP capability of the instrument [106].

The group's library building strategy consisted in narrowing down the criteria normally present in commercial libraries to only molecular formulae, that provide modern high resolution mass spectrometers with information to calculate intended AM and isotopic pattern [106].

After testing the library in real *postmortem* cases from urine, blood and hair samples, the team reported that due to the large quantity of compounds in the database, to each

spectrographic peak it could be appointed 1 to 39 identical molecular formulae from those present in the database [106].

Concluding, in none of their samples a compound could be unambiguously identified due to lack of discrimination between isomers. This leaded to the assumption that AM and isotope pattern alone are not enough for identification purposes and additional criteria should be provided in the database. As for that, the same research group purposed a "metabolomics" approach to shorten the compound hit list with the addition of a criterion that regarded the presence of metabolite [3, 106].

This complementary approach used 108 compounds from the same generous database of 50500 compounds from the previous research. Those selected compounds were proved to be present in the *postmortem* cases by parallel LC-TOF-MS or CE-TOF-MS trials [3].

With resource to a software tool "*E-Dragon software*" it was possible to correlate AMs to pre-defined biotransformations (i.e. hydroxylation, oxidation, demethylation, glucoridation, etc.) that are commonplace in metabolism phase I and II [3].

This permitted to screen for the 108 compounds plus the major metabolites for each parent compound. Hence, while rescreening the samples, the group proved that the simultaneous presence of the parent compound and metabolite molecular formulae reduced the average number of hit for each peak present in the spectrum [3].

On the other hand, the still considerable amount of possible hits for each molecular formulae failed to provide unambiguous results. So the author concluded that it is necessary to introduce another criterion for the search strategy and suggested a theoretical calculation based on physicochemical properties of the molecules in order to obtain an *in silico* RT [3].

Despite not providing any unambiguous results, these two last papers made a statement of the advantages that HR/AM provides. It allows a much broader screening for PTRCs than screening approaches based on library search on product ion mass spectra databases. Additionally, technological development (especially in computation performance) that took place since the publication of these 2 researches, allows today the storage of information rich TICs and respective spectra information of all chemical intervenients capable of being detected by HR/MS [6, 109].

With a comparatively less ambitious database Lee, *et al*. [2] took part in an inter-laboratory research involving 6 investigation groups (including RKA´s). Their method used different instrumentation than the one used in this thesis, but it applied the same methodology concept. A "real-time" acquisition of low and high fragmentation spectra in the same analysis was described in their study [2].

It used a database containing monoisotopic exact mass, RT and nominal fragmentation pattern. Accordingly to the author, with the latter it was possible to attain an optimal fragmentation by increasing the voltage on Aperture 1 (located within the transfer optics of the instrument ion source) from 10 eV to 45 eV. An example for 2 chemically distinct analytes is present in **Figure 1.32** [2].

Function 1 spectra were collected under low energy fragmentation at mild conditions within the source region and provided information of the intact protonated molecular species. Function 2 spectra ware collected using higher fragmentation conditions. Both fragmentation functions were provided by ESI in positive mode [2].

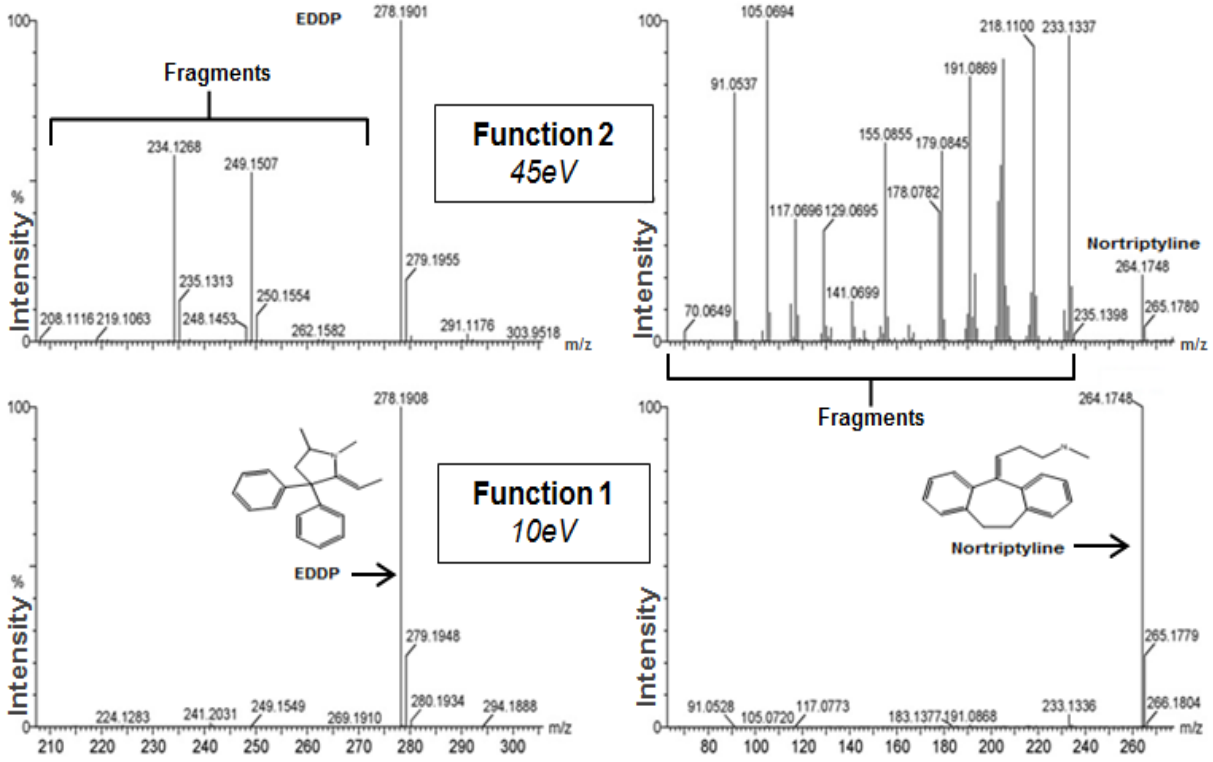

Figure 1.32 *Function 1 and 2 spectra for EDDP and Nortriptyline [2].*

A third spectra acquisition function (not illustrated) of the approach developed in their study, implied the recording of data of the reference mass of leucine enkphanin, which was used to verify mass accuracy throughout Lee *et al.* research [2].

For better specificity, the group took full advantage of exact mass determination and the ability to measure the mass of an analyte to four decimal places, which provided a very high confidence of identification and a small amount of peaks to consider in the analysis. However their work failed to provide adequate sensitivity to the method, even after attempts of converting the data (recorded in monoisotopic exact mass) to nominal mass. Which accordingly to the author, would minimize the possibility of analytes being excluded (or missed altogether), in the eventuality of their exact masses being compromised. However, in the end, this conversion applicability wasn't successful [2].

Another example of STA based on HR/AM acquisition and DIA was the research of Pedersen, *et al*. [6], which took place in the RKA laboratories. The UPLC-(HR)TOF-MS instrument used in the research was one edition older than the one used in this thesis experiment. Their targeted screening procedure was very similar to the one used in this present method and the validation of Pedersen´s methodology was of most importance for comparative reason with this project [6].

Their database was constituted by 5 internal standards (IS)s and 256 PTRCs. The information stored in their method´s targeted library was the same of that employed in this project´s targeted compound database: parent ion exact mass, isotope pattern, theoretical RT, and exact mass of the most abundant fragment ions for each analyte (limited to the a maximal fragmentation voltage of 40 eV) [6].

The author carried out a representative number of STAs comprising 1335 authentic forensic traffic cases by screening for the intended 256 drugs.

DIA is nearly always associated with HR/AM acquisition [2, 6, 75-77, 109]. However, Humbert, *et al*. [62] proposed a GUS based on DIA by gathering parent and product ion spectra (nominal mass) for further correlation with a targeted library containing itself spectral data [62].

The GUS comprehended 500 PTRCs, a target database with 2975 spectra, and used a UPLC-MS/MS for a 15 min cycle analysis. For each run, ESI functioned as a positive and negative ion source. To each polarity different cone voltages were applied, resulting in the collection of 6 different product ion spectrums for each targeted PTRC. Consequently, each intended compound could be characterized by a combination of two criteria: RT and up to a maximum of 12 spectra (combination of $[M+H]^+$ and $[M-H]^-$ modes) [62].

Despite “DIA” has not been mentioned in this literature, Humbert´s methodology suggests this acquisition mode usage as it was reported that, for each single run, multi-acquisition data were acquired in full scan mode from 80 to 650 m/z and the scan speed was around 7000 Da per second. For instance, scans with positive voltage in the ion source cone covered the CID energies of +20 V, +35 V, +50 V, +65 V, +80 V and +95 V. For negative polarity the applied CID energies where symmetrically homologous [62].

This was a possibility with regard to the software (*ChromaLynx*™) capability of preforming an automated integration of the component peaks at each discrete cone voltage (see **Figure 1.33**). *ChromaLynx*™ also permitted the application of deconvolution techniques for removal of non-specific background ions from the spectra [62].

The figure exemplifies oxazepam (a metabolite of diazepam) which ionizes in both positive and negative CID polarities, yielding fragmentation data for the maximum 12 different cone voltages. In both charges it is possible to attain the disappearance of the

parent ion, as the fragmentation pattern for the compound is revealed as the energy increases [62].

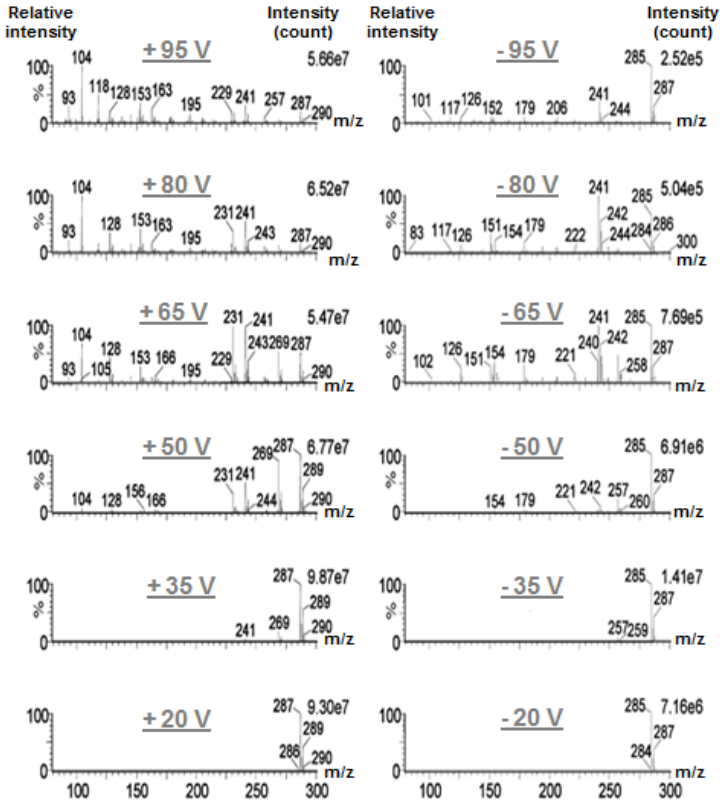

Figure 1.33 *Spectra of oxazepam in positive and negative CIDs (left and right, respectively)* [62].

Additionally, and enforcing the statement of DIA, Humbert, *et al*. [62] concluded that in contrast to classical MS/MS screenings, the full scan approach (the same used in this thesis) can produce a wealth of dimensions for unambiguous identification of unknowns. In opposition, triple quadruple screening monitors for previously selected protonated molecular species (known as qualifiers). Then a DDA mode is activated, but normally it is limited to acquisition of solely 2 product ions [62].

Notwithstanding, the main disadvantage of their method raised when candidate (metabolite or drug candidate) structures were to be structurally elucidated, despite the author´s estimation that their method would be able to provide candidate structure based on 5 to 8 fragments. However the researcher concluded that, for unambiguous identification of a complete unknown in a non-targeted screening, in addition to fragmentation pattern, it was needed accurate mass acquisition [62].

Paul, *et al*. [109] is probably one of the most recent papers to cover a screening method for identification of unknown peaks. The research employed a UPLC-qTOF-MS and performed a GUS based on a HR/AM acquisition with application of non-targeted DDA. The biological matrix of relevance was urine [109].

As their screening was based on DDA, the qTOF device was used to its full capacity. So the first quadrupole served for isolation of precursor ions in DDA mode (auto-MS) and a linear hexapole collision cell (with N_2 as collision gas) was used for precursor ion fragmentation. The method was applied to 76 real case urine samples [109].

Their screening was divided into two. The first was a MS scan for precursor ions and the second scan produced MS/MS spectra rich in fragment ions. The 2nd cycle was mediated upon the data acquisition of targeted parent ions in the MS scan. However, this was already reported in works such as of Broecker, Lafaye, Muller, Rosano, and Paul [1, 7, 83, 101, 109]. The

innovative mark left by Paul *et al.* study was the fact that it goes beyond the simple acquisition in data-dependent auto-MS mode.

When a standard screening with auto-MS is adopted, data is generated statistically and is dependent on strict criteria, like precursor ion abundances, making the resulting spectra of targeted screening often complicated to interpret.[1]. Given that and the fact that the author was searching mainly for unknown metabolites, in the referred research it was combined a first screening based on precursor abundance of 49 compounds present in a "preferred" targeted list, with a data-dependent MS/MS spectra generation of even not listed targets (i.e. completely unknown metabolites). The latter MS/MS scan was not based upon precursor ion abundance and as for that the product ion spectra provided broad fragmentation pattern information as seen in **Figure 1.34**. There, graphic information exemplifies the approach used by author to identify unknown metabolites, by utilizing to the fullest the instrument´s HR/AM acquisition capacity [109].

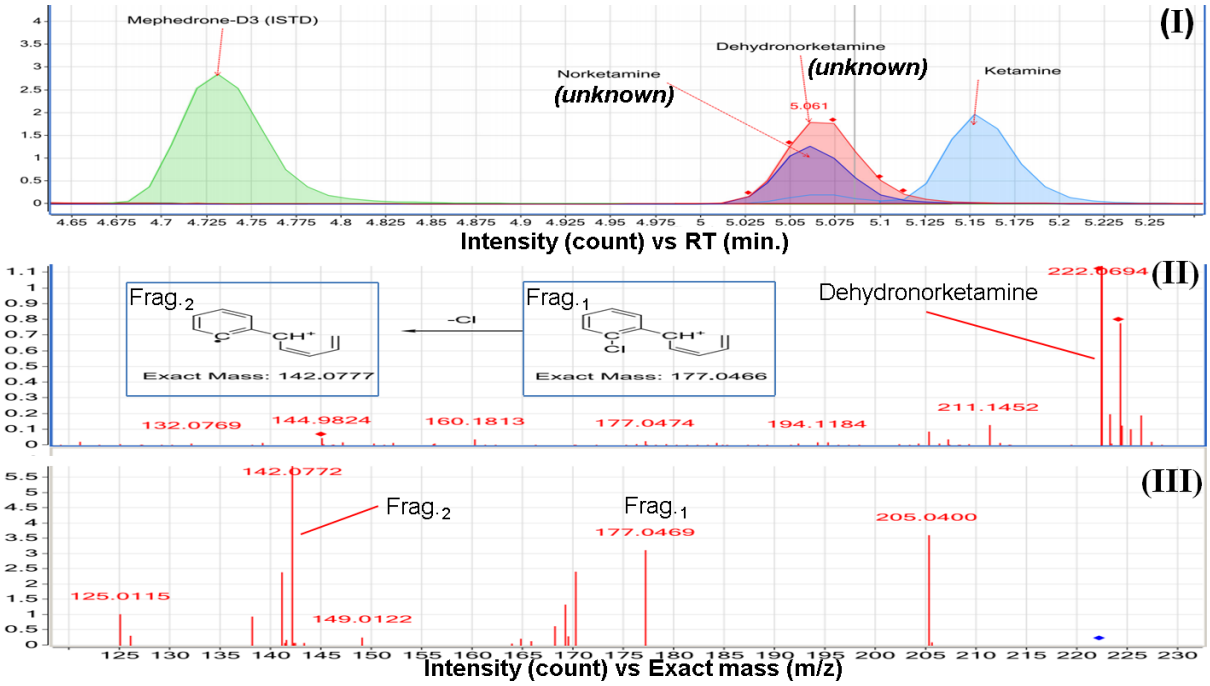

Figure 1.34 **(I)** *Extracted chromatogram displaying an IS (mephedrone-D3), ketamine and 2 coeluting unkown metabolites that proved to be norketamine and dehydronorketamine;* **(II)** *MS precursor ion scan for dehydronorketamine* **(III)** *MS/MS product scan for dehydronorketamine* [109].

The figure demonstrates that even without possession of the corresponding metabolite´s pure standard, the DDA method was able to deliver data thereof. In fact, in **Figure 1.34** (**I**) it was attained the presence of an unknown peak that was found to be a co-elution of two unknown compounds. To structure elucidate these peaks, MS/MS spectra was took at where the red rhombuses are assigned in **Figure 1.34** (**I**). Exemplary, MS/MS spectra of dehydronorketamine was recorded and displayed in spectrums **Figure 1.34 (II)** and **Figure 1.34 (III).** In this case the MS/MS spectrum was recorded though the metabolite was not listed in the preferred table of the data acquisition tool [109].

Guided by literature information about expected metabolites of ketamine, the author was able to identify a fragmentation pattern that put in evidence the presence of the referred metabolite. Hence, it was proposed a fragmentation pathway for the MS/MS spectrum of dehydronorketiapine (see **Figure 1.34 (III)**. The base peak at m/z 142.0772 ($Frag._2$) was explained by the elimination of a chlorine radical from m/z 177.0469 ($Frag._1$). This way, two fragments were able to be identified, which enhanced the structure elucidation of the peak eluted at 5.061 min (**Figure 1.34 (I)** [109].

A similar approach was used for the structure elucidation of norketamine (spectra not available) [109].

Concluding, Paul *et al* approach was able to ensure the positive identification of ketamine and its metabolites in urine, even without the use of expensive pure standards [109]

This review cannot be completed without summarizing the vast work produced by the group of Ojamperä [73,102,110-112].

The first to apply the concept of high-resolution mass spectrometry to general unknown screening in forensic toxicology, Ojamperä*, et al.* [110] primordial procedure involved a database assembling containing theoretical accurate masses of toxicologically relevant compounds and for some cases, their respective metabolites [110].

After their early approaches, the author concluded the necessity of including RT [111], metabolite exact mass [66, 97] and most importantly, additional structure information of compounds, provided by CID fragmentation spectra [73, 112].

Despite all their work being based on HR/MS acquisition, none of the researchers employed DIA, failing to explore this new capability for STA.

2 EXPERIMENTAL

2.1 Solutions

Ultrapure water was prepared with a Milli-Q system (Millipore, Denmark). MeOH and ACN (LC-MS grade) were obtained from Fisher Scientific (Leicester, United Kingdom). Ammonium formate was supplied by Sigma-Aldrich (Steinheim, Germany).

All reference compounds, present in BAMBS, TOF mix 1 and TOF mix 2 were purchased from Lipomed GmbH (Bad Säckingen, Germany), Cerilliant, (Round Rock, Texas, USA), or Toronto Research Chemicals (Toronto,Canada). BAMBS stands for Beta-agonister Astma Misbrugsstoffer Benzodiazepiner Specielle (Beta-agonists Asthma Drugs of abuse Benzodiazepines Special, in english). Additionally, the preservative sodium fluoride was purchased from BD (Plymouth, United Kingdom).

Purity of the reference standards was ≥ 98%. All 5 deutered ISs, amphetamine-D5, diazepam-D5, morphine-D6, methadone-D3, and mianserine-D3 were purchased from Cerillant (Round Rock, Texas, USA).

Two mixtures, TOF mix A and TOF mix B, containing 98 and 100 PTRCs respectively were prepared as a stock solution in MeOH, yielding a concentration of 10 mg/L. From the stock, working solutions were acquired for further laboratory application by dilution in 50% MeOH in water. The stock solution was stored at -80 °C and the diluted working solutions (50, 100, 500, 1,000 and 5,000 ng/mL) were stored at -25 °C.

A single solution containing all 5 IS was prepared in MeOH, with an individual final concentration ranging from 0.1 to 4 ng/mL. The solution was divided in ampoules of 1 mL and stored at -80°C.

4 stock solution in MeOH of 4 designer drugs (Pyrovalerone, 5-FUR-144, 5-F-AKB48, α-PVP), each at a concentration of 1.0 mg/mL were stored at -20ºC.

Stock solution in MeOH of E-OH-nortriptyline and Z-OH-nortriptyline, each at a concentration of 1.0 mg/mL and stored at -20ºC.

Stock solution in MeOH of 7-OH-quetiapine at a concentration of 1.0 mg/mL, stored at -20 ºC.

2.2 Materials

Strictly for blank samples, whole blood was obtained from the Blood Bank at the Copenhagen University Hospital (Copenhagen, Denmark). The biological matrix was preserved by the RKA staff with 1 % sodium fluoride. The blood will subsequently be referred to as blind blood. Additionally and for the same effect, *antemortem* and *postmortem* blood was also acquired from archived and compound negative forensic cases. Storage was made at -20 °C until use.

Whole blood samples acquired from autopsied and living persons, implicated in open and closed forensic cases, were analyzed.

2.3 Method

2.3.1 Sample Preparation

The sample preparation of choice was PPT and the liquid handling, which included pipetting steps, centrifugation and evaporation, was entirely performed by a Freedom EVO 200 robot from Tecan Group Lts (Männedorf, Switzerland).

The procedure within the instrument was as follows:

(1) From each sample's total whole blood, the liquid handler transferred 100 mg to a hole in a 96 well plate;
(2) One ampoule with the IS solution, referred in **section** Erro! A origem da referência não foi encontrada., was added to each sample of 100 mg by, the liquid handler;
(3) 700 µL of ACN was added and further shacking precipitated the matrix;
(4) After precipitate formation, a centrifugation step at 3600 rotations per minute (rpm) for 10 minutes took place;
(5) Further pipetting separated the supernatant from the denser protein precipitate;
(6) In a new vial the supernatant's organic solvent molecules were evaporated by a stream of nitrogen at a temperature of 35 °C, until container was free of moisture;

(7) In the dried vial, reconstitution was attained by addition of 0.1 mL of 25 % MeOH in water containing 0.1 % of formic acid and the solution was shacked;
(8) The resulting solution was then transferred for a 96 well plate. At this point the sample was ready for chromatography injection, which had a volume of 5 µL.

2.3.2 Liquid Chromatography

The chromatography was performed using a Waters Acquity UPLC system from Waters Corporation (Milford, MA, USA).

The instrument setup was based on the same employed by Humbert, *et al*. [62], Rosano, *et al*. [101] and application notes provided by Waters™.

Separation was performed in a hollow structure section (HSS) C_{18} column (150 mm x 2.1 mm x 1.8 µm) from Waters™ and maintained at 50 ºC, with an uniform flow rate of 0.4 mL/min.

During the UPLC binary solvent run, the mobile phase was composed by a gradient of 2 solvents. Solvent A was constituted by an aqueous solution ammonium formate with concentration of 5 mM. pH was adjusted to 3 by formic acid addition. Solvent B was composed by 0.1% (v/v) formic acid in ACN.

Table 2.1 provides the binary solvent composition during the 15 minutes chromatographic run.

Table 2.1 *UPLC gradient over time.*

Time (min)	Solvent B (%)
0.00-0.5	13
0.5-10	13-50
10-10.75	50-95
10.75-12.25	95
12.25-15	13

2.3.3 Mass Spectrometer

The ion source/analyzer/detector used in this thesis was a Xevo G2-S QTOF from Waters (Waters MS Technologies, Manchester, United Kingdom). The detection type involves charged particles orthogonal acceleration and measurement of their “time-of-flight”.

Exclusively positive mode ESI (z-spray) was employed, although negative mode is an option provided by the instrument. Ion source settings were as follows: capillary/aperture voltage was set to 800 V; voltage applied at the cone was 25 V; nebulation gas flow rate was

1000 L/h at a temperature of 400 °C; cone gas flow was set to 10 L/h; argon was used as collision gas; and the overall temperature at the ion source chamber was 150 °C.

As highlighted in **section 1.4.6** a DIA experiment, trademarked as MS^e by the instruments manufacturer, was delineated. At this stage two different collision energies functions (LE and HE) were used in the collision cell of the analyzer. The fragmentation at LE function was effectuated at 4 eV whereas HE function involved a ramping from 10 to 40 eV.

The operation resolution was set to be higher than 18000 at FWHM. For resolution measurement and assurance of reproducibility and accuracy, a LockSpray™ was utilized. Leucine-enkephalin parent ion with 556.2766 m/z was used as lock mass. The LockSpray™ solution with a concentration of 400 ng/mL and a spraying flow rate of 10 µL/min was used for all analysis.

Data acquisition was performed in continuous mode and encompassed the mass interval from 50 to 950 m/z. In the detector, for both LE and HE functions, each data channel collection was performed at 0.20 s/scan, intercalated by an inter-scan delay of 0.024 seconds. Hence, each DIA cycle lasted 0.424 seconds. Relatively to the total 15 min chromatography run time, data acquisition started at minute 0.9 and lasted until minute 13.

2.4 Software

2.4.1 *UNIFI1.7*™

The data processing software employed in this project was *UNIFI1.7* (Waters Corporation, Milford, Massachusetts, USA). This tool performed data acquisition and storage for posterior analysis. Dependently of the screening method applied (targeted, semi-targeted or non-targeted) several parameters were altered accordingly to the circumstances. In this section it is discussed the used databases for unknown identification or for candidate structure elucidation. Additionally, it is highlighted the criteria that directly influenced the overall sensitive and specificity of the screening. Both varied accordingly to the amount of available data about what was to be screened for.

Hence, in targeted screening the identification criteria were: exact mass < 3 mDa (mass error ± 3 mDa); experimentally acquired RT within ± 0.45 min from the library value provided (RT error) and a response threshold down to 114. The targeted database of compounds utilized was a Waters Toxicology Library™ (Waters Corporation, Milford, Massachusetts, USA) containing, among other information the molecular formula (exact mass and isotope pattern) of 1030 parent ions. Additionally, for each compound the respective product ions exact mass (if applicable) was also provided.

In semi-targeted screening, criteria that define the listing of the suspected hits in the "analysis method" compound list were as follows: mass error ±3 mDa; and response threshold up to 1000. The employed library (see, **section 3.2.1**) was a 1392 compound database in-house assembled storing, between other parameters: common name; molecular formula (exact mass) of compound and fragmentation pattern obtained either by direct exact mass plotting or through importation of molecular structure. The feasibility of these last two approaches is discussed in **section 3.2.3**.

Non-targeted screening identification criteria corresponded to a mass error of ± 3 mDa and to a higher response threshold of 30,000. No internal library of compounds was used. The external and online reference libraries used for structure elucidation were: *ChemSpider* (Royal Society of Chemistry, UK) and *m/zCloud* (HighChem LLC, Slovalkia).

2.4.2 *ACD/MS Fragmenter™*

Fragmentation prediction based on *in silico* conditions for HE collision functions was executed by *ACD/MS Fragmenter 11.01* from Advanced Chemistry Development laboratories (ACD/labs, Toronto, Canada). The software is a fragmentation prediction program based on established MS fragmentation rules from literature. For each predicted fragment it produced a three-structured presentation. Additionally this tool provided detailed information on the routes of fragmentation and all possible structure candidates for a specific mass. The exact masses of the fragments were provided automatically [97].

The settings adopted for this study were as follows:

- In this context, the ionization type selected was API (ESI) in positive mode;
- Charges from multicomponent structures and processing of salts were removed when possible;
- Only hydrogen shift was allowed in the prediction, in detriment of double bonds cleavage, triple bond cleavage and saturated rings cleavage;
- The reactions took into account for fragment formation were resonance and hydride shifts, as ring formation was not considered;
- Exclusively acyclic bonds were selected to be cleaved;
- The "Maximum Fragments Generated on Each Step" was set to 10;
- The "Number of Fragmentation Steps" was set to 2;
- The "Minimum Mass Value" for a fragment to be displayed was of 50 m/z.

2.4.3 Additional software

Fragmentation studies prediction and molecular structure drawing were performed by *ChemBioDraw Ultra 12.0* (CambridgeSoft, Cambridge, Massachusetts, USA).

2.5 Databases

2.5.1 LIMS

LIMS is an in-house built RKA database which conciliates the results of several LC-MS/MS and TOF/MS methods for each sample analyzed. The LC-MS/MS methods perform: quantification of barbiturates in hair; quantification of antipsychotics; quantification of exotic drugs; quantification of drugs present in very low dosages; and quantification of all common PTRCs such as amphetamines, opiates, opioids, benzodiazepines (see **section 1.3.1**). Additionally, a Q Exactive™ is used to screen for synthetic cannabinoids.

The other non-LC-MS/MS instrument that contributes for the results provided in the LIMS corresponds to the previous version of the TOF/MS instrument employed in this thesis and it screens exclusively for barbiturates, and its ionization polarity is set in negative mode.

2.5.2 *ChemSpider™*

This on-line free chemical structure database provides a fast and free access to over 32 million structures, properties, and associated information. It integrates and links compounds from approximately 500 data sources [113].

2.5.3 *mzCloud™*

A mass spectral database provided in Internet containing a freely searchable collection of accurate mass spectra. This was used for analytically assist on the identification of library absent compounds [114].

This tool was used in the last stage of non-targeted screening. It was used to search for m/z by correlating the analytically acquired three most intense product ion peaks of intended unknown compounds, with the AM spectra present in the on-line database [114].

The “Peak Search” parameters were:

- “Search Type” was “MS^n”;
- “Libraries” field was set to “Reference”;
- “Ionization Mode” imaged the one used in this project and was exclusively set to “positive”;
- The “Search in” field was set to “Filtered Spectra” and “Recalibrated Spectra”;
- “Precursor m/z” was compound dependent;
- “Peak List” report was compound dependent and the “m/z Accuracy” was set to 0.009 Da.

3 RESULTS AND DISCUSSIONS

55 whole blood samples were screened for PTRCs by the three staged method. These samples pertained to *antemortem* and *postmortem* cases analyzed in the RKA between 1 October 2013 to 31 December 2013.

In this chapter all steps of the developed method will be described and explained. The examples will contain a degree of continuation and correlation between the different stages of the method. This is particularly evident between the targeted and the semi-targeted analysis.

Evaluation and case sample analysis procedures will be integrated in this chapter, as there is interdependency between both studies.

3.1 Targeted screening

3.1.1 Detection, Filters and Identification criteria

The interpretation of chromatographic and spectral data using *UNIFI1.7™* was the first step of the analysis. The data treatment was guided by three factors: detection criteria, filtering criteria and identification criteria.

Detection

This factor corresponded to the instrument/software capability of detecting any peak in the analysis. Its sole criterion was that any chromatographic peak would be considered as a hit if its signal was 3 times higher than the baseline noise. Given the high RP of the analytical instrument employed in this project, the detected chromatographic peaks were in the order of the thousands *per* injection. A DIA cycle was performed at each detected peak. The resulting raw chromatograph didn´t display any additional information, such as peak identification.

Filters

In order to narrow down the number of hits in every blood sample´s chromatogram and for effective compound identification, targeted screening had to be implemented. It consisted in limiting the data to be provided in the "Analysis Method" view (see **Figure 1.24**) to that of analytes listed in a targeted database. This process was done by automatically correlating empirically acquired data with information plotted in a commercially acquired list of compounds. The used targeted library contained 1030 compounds and enclosed information such as: compound name, molecular formula, expected RT, molecular structure and exact mass of fragment(s). The fragmentation information was strict to that predicted at a collision energy ramping from 10 to 40 eV. Hence for some analytes there wasn´t present any fragment information, as they fragment at different energies from those applied in this thesis´ DIA function.

The basic filter provided by the instrument is denominated "identified", which contained the following default filtering settings:

- Only compounds present in the targeted database are accounted for in the screening (identification status = identified);
- The difference between the acquired exact mass and the mass stored in the library (mass error) had to be lower than 3 mDa;
- The difference between the acquired RT and the RT stored in the library (RT error) had to be within +/-0.45 min.

However, because of the broad criteria used by the "identified" filter, the FP rate was very high, which hampered a time effective sample analysis. In order to reduce the number of detected compounds that corresponded to FPs, filters with narrower criteria were developed. The filter development study is described in **section 3.1.5**.

Contingent upon the filter employed, there was a variation in the number of compounds to be classified has FP or UP listed in the "Analysis Method" window of the software.

Identification criteria

Identification criteria are what guided the operator to classify each hit as falsely (FP) or truly (UP) present in the sample. The filter criteria varied along the study, however, identification criteria, despite prone to subjectivity, corresponded to a more static set of rules:

- The presence of adducts (Na^+ and K^+) in addition to the protonated (H^+) ion enhanced the hypothesis of the hit to be an UP;
- The exclusive presence of adduct(s) without the presence of the protonated ion in the LE spectrum categorized the hit as a FP;

- The presence of the parent drug and its metabolites in the same sample further regarded the hit as an UP;
- In the same sample, the presence of compounds of the same chemical class or with similar toxicological effects classified the hit as UP;
- The presence of fragments was accounted as the most important parameter to classify the hit as an UP. Especially if the product ion was exclusive to the compound in analysis or if the fragment was shared between a parent drug and its metabolites.

3.1.2 Method evaluation: LOD, LOF and LTC

The fragmentation pattern was of upmost importance for the identification of PTRCs in this thesis. Hence, it was implemented a procedure to empirically attain information about the LOD, LOF and LTC of 198 PTRCs from the targeted library.

LOF corresponded to a narrower re-definition of LOI, as it exclusively provided the approximate blood concentration at which a chemical entity fragments, within collision energies ramping from 10 to 40 eV.

This experiment consisted in spiking samples of 100 mg of blind blood with 198 compounds divided into two mixtures, TOF mix A and TOF mix B, at different concentrations. The assembly of these mixtures was part of a protocol delineated by the RKA in order to implement a comprehensive method that will screen for the 300 most commonly identified PTRCs.

The PTRCs concentrations accounted for were 0.001, 0.002, 0.005, 0.01, 0.02, 0.05 and 0.1 mg/Kg. In order to consistently determine the LOD and LOF values, for each concentration, TOF mix A was spiked into three homologous blind blood samples. Therefore, globally 21 blind whole blood samples were spiked with 98 PTRCs contained in TOF mix A. The same methodology was implemented for TOF mix B, which contained the remaining 100 compounds.

The sole detector response and fragmentation information for each PTRC was acquired by comparison of the three homologous samples´ results. The criteria were as follows:

- For each compound, the response for each concentration was the mean of the three trials;
- If in any one of the three samples, for each concentration, at least one didn't provide fragment(s), the overall result was set to "no fragmentation".

The filter used in this procedure was the “identified” and the resulting table is presented in the **Appendix I**.

Additionally, LTC values were attributed to a representative portion of the compounds present in the table. The values were provided by the literature, with clear relevance to Baselt [21].

The LTC contributed to qualify the importance of AM acquisition of product ions. Additionally it was of upmost importance in the method´s sensitivity evaluation, discussed in **section 3.1.6.**

3.1.3 Sample analysis

The results from the procedure discussed in the previous section revealed that from the 181 compounds, to which LTCs were determined, 43 did not fragment at concentrations below (or at) the LTC. Additionally, 4 out of the total 198 PTRCs weren´t detected and 9 didn´t provide product ions below the highest concentration considered in the study (0.1 mg/Kg).

These results revealed that, despite an important tool for peak identification, fragmentation couldn’t be the sole criterion to be taken into consideration in the analysis. For cases where product ions weren´t provided, the remaining **identification criteria** listed in **section 3.1.1**, had to be considered.

For explanation of the complexity of compound identification or cause of death determination, two examples are discussed in this section **Figure 3.1** and **Figure 3.2**, page 57, are displayed.

Methylphenidate, is a psychostimulant drug used in the treatment of attention-deficit hyperactivity disorder (ADHD), postural orthostatic tachycardia syndrome and narcolepsy [21]. It was classified as a CP hit in sample TIM3052, relative to a *postmortem* case where the cause of death was a phenobarbital overdose**.** The result was provided by both parallel methods employed in RKA and in the method hereby developed. In the latter the presence of a product ion in the HE spectrum was what exclusively elucidated its identification. Hence this is an example of a LOF facilitated compound identification.

Figure 3.1 illustrates the software´s "Analysis Method" view after selection of the methylphenidate´s hit in the "Component Summary".

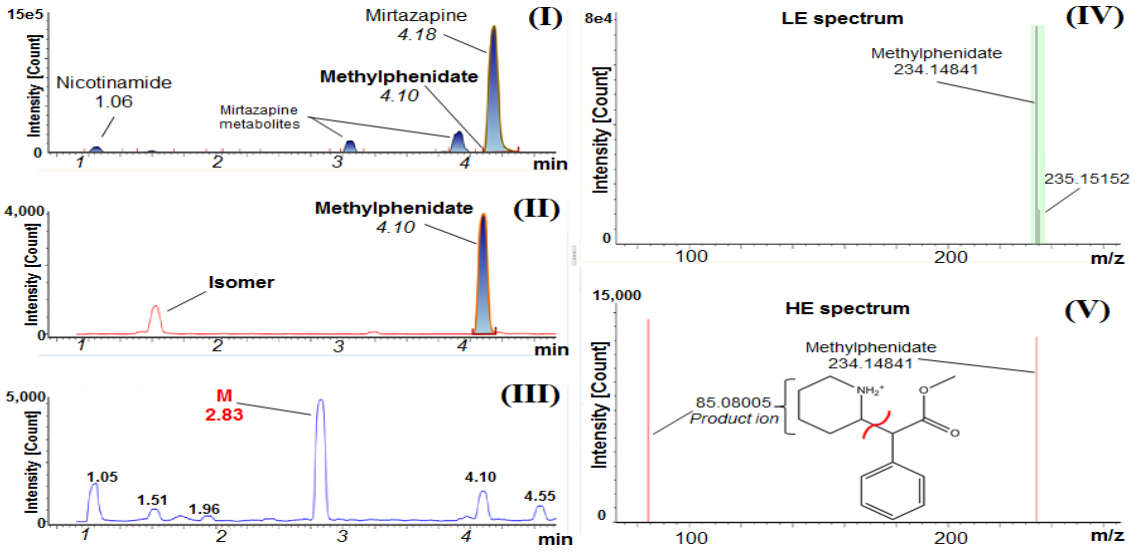

Figure 3.1 *UNIFI1.7™ "Analysis Method" window of detection for methylphenidate in sample TIM3052.* **(I)** *TIC evidencing a discrete methylphenidate elution at 4.10 min;* **(II)** *XIC for the mass 234.14841 m/z, which characterizes the protonated molecule of methylphenidate and a disclosed isomer;* **(III)** *XIC for the mass 85.08005 m/z, not exclusively related to the identified product ion;* **(IV)** *Low collision energy spectrum for the elution time of 4.10 min;* **(V)** *High collision energy spectrum for the chromatographic peak at 4.10 min.*

The subfigure **(I)** is the TIC in which it was possible to account for 4 clearly highlighted peaks correspondent to nicotinamide (1.06 min), mirtazapine (4.18 min) and both its metabolites: 8-hydroxymirtazapine (3.06 min) and N-desmethyl mirtazapine (3.91 min). As the finality of a TIC is to provide a holistic view of all chemical entities present in an injection (i.e sample), it is dependent on the highest count recorded. In this case it corresponded to mirtazapine which peak had an intensity of about $15x10^5$ counts. As this instrument was regarded with a high RP (operated at a resolution higher than 18000 at FWHM) it was possible to detect a peak that eluted in the proximities (4.10 min) and had an intensity several times lower. Most importantly, the software was able to highlight the intended peak in a separate chromatogram.

The chromatogram **(II)** provides the XIC for the exact mass of 234.14841 m/z, which corresponded to that of methylphenidate. However, this software capability is not compound specific but it aims for a specific AM. Hence, in this case, it displayed the methylphenidate peak but also an isomer that eluted at around 1.5 minutes or the compound that elutes at 1.5 min had a fragment with the same exact mass of methylphenidate, which is highly improbable. Either way, the peak belonged to a compound not listed in the targeted library so it remained *incognito* throughout the analysis. Despite of not providing relevant information for the STA, with this example of isomer identification, it was possible to explain the feature of *UNIFI1.7™* exact mass appointment tool (introduced in page 27, **Figure 1.28**)

Subfigure **(III)** pertained to an additional XIC, this time specific for the exact mass of 85.08005 m/z that characterized the sole methylphenidate product ion. This fragment was

what made possible the indubitable classification of its precursor ion as an UP. In image **Figure 3.1 (III)** the RT of this fragment didn´t exclusively correspond to the elution time of its precursor ion (methylphenidate), but also to at least 7 additional RTs. This means that 7 other compounds in the sample produced a fragment with an exact mass of 85.08005 m/z. As similarity of fragmentation pattern indicates similarity of molecular structure, this comparison tool proved useful for the identification of transformation products (TP) or metabolites, which presence is an active part of the **identification criteria** referred in **section 3.1.1**. Notably, in this XIC there is a compound signaled as "M", with a RT of 2.83 min, its importance for the STA will be addressed in **section 3.2.2**.

Figure 3.1 (IV), depicts the spectrum resulted after fragmentation of the PTRC eluted at 4.10 min. The collision energy employed was 4 eV and corresponded to the LE function of the DIA. There it is illustrated a single peak with mass of 234.14841 m/z and one of its isotope, smaller in intensity, with a mass of 235.15152 m/z. In this case of methylphenidate, it wasn´t detected any adduct (Na^+ or K^+). However, they can be detected both in the LE and HE spectra and are an integrant part of the **identification criteria** listed in **section 3.1.1.**

Adducts were produced in the method´s ion source and were formed from the analyzed precursor ions. The ions preserved all its constituent atoms but instead of a protonation, it was added one of the two referred possible atoms. Their importance resided in the fact that, with more ionized species for a single PTRC, more parameters were collected for the intended compound identification [115].

Figure 3.1 (V) presents the high collision energy spectrum for methylphenidate´s RT, the final stage of a DIA cycle. The collision energies involved corresponded to a ramping starting at 10 eV and finalizing at 40 eV. It provided the most valuable information for compound characterization: its fragment(s) exact mass(es). In this case, from the peaks present in the LE spectrum, *UNIFI1.7™* exclusively revealed the peak with 235.15152 m/z, which had, from the previous spectrogram, decreased from $8x10^5$ to about 15000 counts. Additionally, it presented the methylphenidate sole fragment with an exact mass of 85.08005 m/z, which corresponded to that of the protonated pyridine, which is one of the substituents of methylphenidate´s phenethylamine backbone structure (see **section 1.3.2.2**).

In **Figure 3.1 (IV)**, it is presented the molecular structure of the analyzed PTRC. At this stage *UNIF1.7™* does not display any structure in the HE spectrum by itself, as the structure was there introduced for reader elucidation reasons.

The provision of fragmentation for methylphenidate (highlighted in **Table 3.1**) was predicted by the method evaluation study referred in **section 3.1.2**.

Table 3.1 *LOI, LOD and LTC information for 6 PTRCs present in* ***Figure 6.I-6.5, Appendix I****, with methylphenidate in evidence. Green background corresponds to fragmentation provision and red background to absence of product ion(s). The number zero symbolizes lack of detection.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
120	Methotrexate	NA	NA	0.04	0	0	0	0	0	0	0
121	Methoxymethamphetamine (PMMA)	0,005	0,005	N/A	0	0	548	941	2015	4396	7889
122	Methylphenidate	0,001	0,002	0.01	225	545	1209	2997	5587	12334	27616
123	Metoclopramide	0,001	<0.001	0.05	261	649	1397	3321	6604	14589	32219
124	Metoprolol	0,001	0,01	0.035	345	809	1803	3979	7694	16592	32491
125	Mianserine	0,001	<0.001	0.015	339	826	1682	4254	7985	17894	36756

The table indicates that this compound was present in the blood sample TIM3052 at a concentration higher than 0.002 mg/Kg. This conclusion was in congruence with the LIMS database, which listed the compound´s concentration at 0.006 mg/Kg. Still, it is lower than the compound´s LTC (0.01 mg/Kg), which is the threshold at which methylphenidate manifests its therapeutic properties in the user.

The classification of methylphenidate as UP proved to be simple and efficient. However, cases such as heroin intake determination, exemplified by sample TIM2955, revealed to be troublesome. In this sample, the analysis of a morphine peak, illustrated in **Figure 3.2**, attributed a classification of FP to the hit. However, in the LIMS it was reported the presence of the compound in the sample. Additionally, the preliminary report pointed the cause of death to be methadone poisoning, and that the deceased had a history of drug of abuse consumption. Hence, the probable intake of heroin had to be proved, not only through the identification of morphine´s fragments, but by a wider set of parameters.

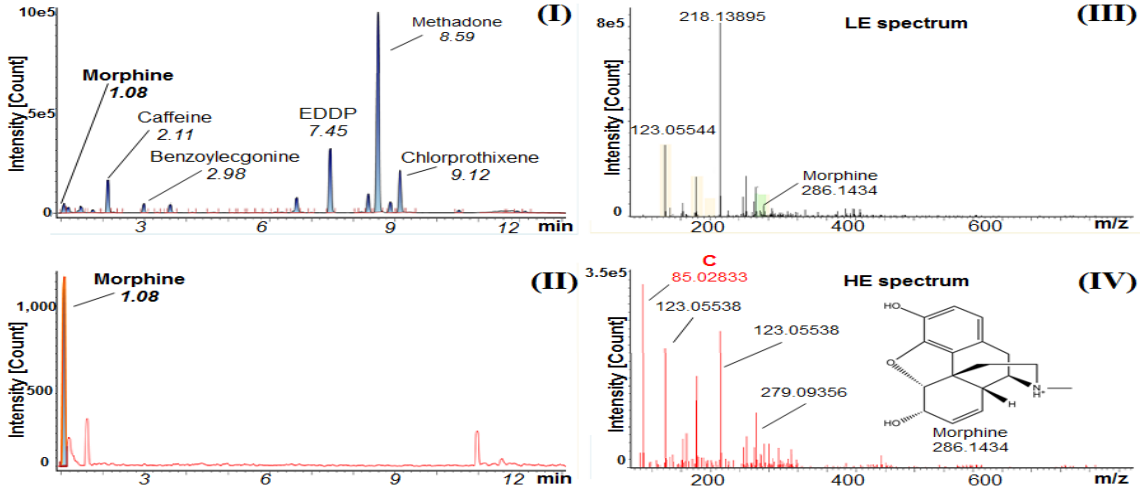

Figure 3.2 *UNIFI1.7™ "Analysis Method" window of detection for morphine in sample TIM2955.* **(I)** *TIC evidencing a discrete morphine elution at 1.08 min;* **(II)** *XIC of the mass of the protonated molecule of morphine (286.14841 m/z);* **(III)** *Low collision energy spectrum for the elution time of 1.08 min;* **(IV)** *High collision energy spectrum for the chromatographic peak at 1.08 min.*

Morphine is the main psychoactive chemical present in opium and one of the active TPs of heroin (see **section 1.3.1.2**) and as stated, it was regarded as a FN in the targeted screening of sample TIM2955.

In the **Figure 3.2 (I)** it is presented the TIC in which it is plotted a low intensity peak relative to morphine, eluting at 1.08 min. Additionally it presents several high intensity peaks. Among them two belonged to the direct causers of death: methadone (eluted at the minute 8.59) and EDDP, its toxicologically active phase I metabolite (eluted at 7.45 min) [21].

Of relevance it is the fact that morphine has a chromatographic early elution, which was an impediment of convenient detection, as there it is present a high degree of ME [71].

The XIC illustrated in the **Figure 3.2 (II)** clearly displays the problematic peak for the exact mass of 286.1434 m/z that belonged to morphine (1.08 min). The remaining peaks on the XIC were discarded as isomers, as their shape and intensity were not satisfactory. Supplementary, as no fragment was detected in this analysis, no additional XIC was presented in this targeted screening "Analysis Method" window.

Figure 3.2 (III) provides the LE spectrum for the RT of 1.08, where the most intense stick peaks corresponded to 218.13895 m/z and 123.05544 m/z, with that of morphine (286.1434 m/z) being relatively residual. The amount of spectrographic peaks in the spectrum resulted from the LE collision energy revealed a problematic detection by the TOF/MS. This supports the assumption of the presence of contaminants at an early stage of the chromatography.

Figure 3.2 (IV), respective to the HE spectrum, didn´t revealed any product ion of morphine, but exclusively peaks related to the fragmentation of the interfering compounds referred in the previous paragraph. One of them had a peak intensity of approximately $3.5x10^5$ and a mass of 85.02833 m/z, signaled as "C". This fragment was accounted as the most common unknown fragment throughout this thesis, and its structure elucidation was covered by the non-targeted screening (see **section 3.3.3)**.

Additionally, morphine has a high molecular stability, evidenced by its richness in aromatic and non-saturated bonds, as observable in the molecular structure, in **Figure 3.2 (IV)**, (again, at this stage molecular structure is not provided by *UNIFI1.7™* and the illustration is exclusively presented for elucidation purposes). This signified that, for fragment provision, it would be necessary higher fragmentation energies at the DIA´s HE function (> 40 eV) or a higher concentration of morphine in the sample TIM2955.

This comes in alignment with the results enclosed in **Appendix I**, from which **Table 3.2** provides a comprehensive extract for analysis of the morphine hit.

Table 3.2 *LOI, LOD and LTC information for 6 PTRCs present in* ***Figure 6.1-6.6****,* ***Appendix I****, with morphine in evidence. Green background corresponds to fragmentation provision and red background to absence of product ion(s). The number zero symbolizes the deficiency of detection.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
129	Morphine	0,005	>0.1	0.01	0	0	77	239	409	939	2159
130	Morphine, 6-monoacetyl	0,001	0,01	0.01	260	599	1353	3107	6066	13072	25795
131	m-Trifluorophenylpiperazine (TFMPP)	0,002	0,002	N/A	0	375	991	1634	3550	8629	15662
132	Naloxone	0,001	0,01	0.01	26	52	222	853	1709	3882	8372
133	Naltrexone	0,002	0,002	0.003	0	429	1121	1881	3829	9197	17248
134	Nitrazepam	0,005	0,05	0.03	0	0	28	123	207	501	1107

The LIMS listed morphine in sample TIM2955 at a concentration of 0.034 mg/Kg, which is above of its LTC of 0.01 mg/Kg and **Table 3.2** reveals that *UNIFI1.7™* didn´t provide product ions for the concentration attained by parallel quantitative methods. Nevertheless, the same table plots "morphine, 6-monoacetyl" (6-MAM), the other active heroin´s phase I metabolite (see **section 1.3.1.2**), as a fragment provider when at a concentration equal or above to its LTC of 0.01 mg/Kg. However, 6-MAM´s presence wasn´t reported by the LIMS, so its concentration remained non-determined throughout the analysis. Hence, this additional heroin metabolite is exclusively present in *UNIFI1.7™´*s targeted list and the "Component Summary" of morphine (see **Figure 3.3**) revealed the presence of 6-MAM in the *postmortem* sample TIM2955.

As demonstrated in the image, 6-MAM, with a "Response" of 151 provided one "Identified High Energy Fragment" which had a mass of 268.13323 m/z. This signified that the compound was present in the sample at a concentration higher than 0.01 mg/Kg. Hence, 6-MAM was classified as an UP, which was used as evidence that the deceased had consumed heroin, despite the cause of death wasn't attributed to that fact.

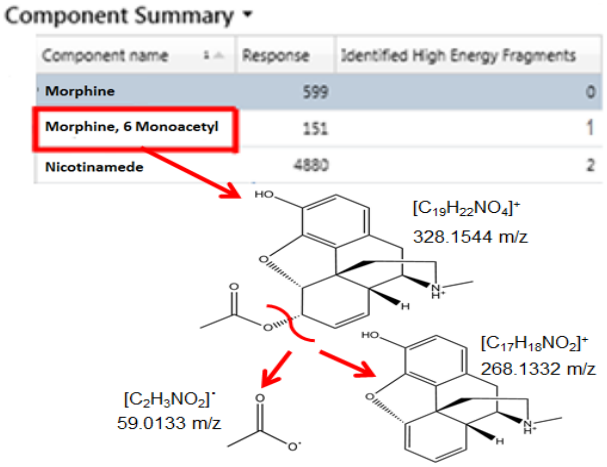

Figure 3.3 *Extract of the "Component Summary" table for sample TIM2955, evidencing the presence 6-MAM, and its most common fragmentation pathway.*

The fragmentation of 6-MAM can be explained by the weak sigma bound between the carbon no. 6 of the backbone structure and the reactive oxygen of the acetate group. There oxygen's electronegativity, fueled by the electron attraction from the rest of the acetyl radical, contributed to a facilitated bound cleavage. This way, at a collision energy of 40 eV and even hypothesizing that 6-MAM is at a lower concentration than morphine, the first exhibited a fragment, which was of interest for compound identification, while the latter, with a more stable structure, didn´t produce any product ion.

Concluding, the parameters which morphine´s hit missed to satisfy, to be classified as an UP, were those that permitted the identification of 6-MAM. This rectified the FN classification of morphine.

Both analyses exemplified in **Figure 3.1** and **Figure 3.2**, provided an explanation of the line of thought followed for the analysis of the 55 whole blood samples. The results are displayed and discussed in **section 3.1.6**.

3.1.4 "Excluded" library

In order to decrease the FPs from the component plot of the "Analysis Method" it was assembled an "excluded" library which consisted in listing compounds with minor toxicological relevancy but with great contribution for a high FP frequency. Those compounds that are present in a substantial number of samples of a batch are to be considered excluded. Compounds like tryptophan which "Summary Plot" is presented in **Figure 3.4**, evidenced that they are considerable expendable for the current STA. In the figure it is shown that tryptophan was present in every blood based injection. The two exceptions were the injections correspondent to "BAMBS 1" and "BAMBS 2" which did not produced any response for tryptophan, as they were aqueous solution. BAMBS consisted in a mixture containing 64 pure standards of pertinent compounds used by RKA in routine analysis. Then the BAMBS reference solution was substituted by TOF mix A and TOF mix B, tested in **section 3.1.2**.

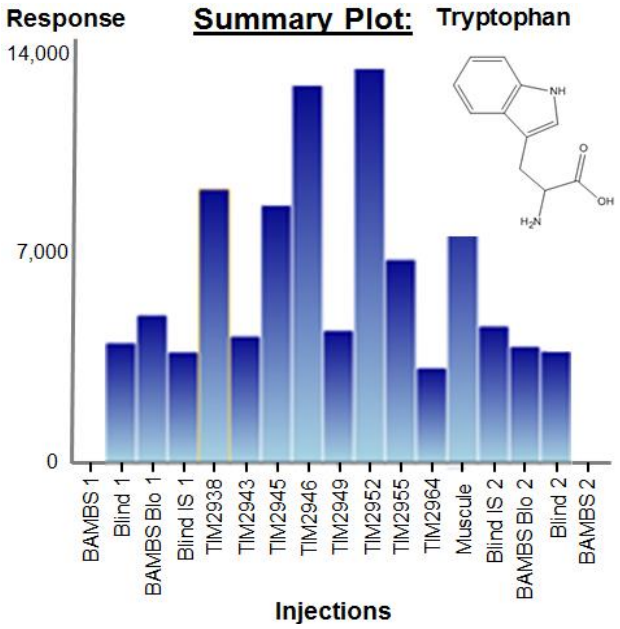

Figure 3.4 *"Summary Plot" of tryptophan detector response in batch 1.*

Differently, in **Figure 3.4**, all the other blood based injections of batch 1 produced a response for tryptophan. The most intriguing were the injections in which it was used blind blood, which produced a response of about 5000. This way, it was concluded that tryptophan was an endogenous chemical of whole blood [21]. However it´s response increased in injection pertaining to cases where the amount of UP for drugs (medical and of abuse) was high. Such as samples: TIM2943, TIM2946, TIM2952 and TIM2955.

This procedure was repeated to all compounds of the 1030 that had produced at least one hit in one of the 6 analyzed batches with the "Identified" filter. Additionally, based on the literature [21], toxicological information about the compounds was listed. All the information

was conjugated in an "excluded" library, present in the **Appendix II**, being **Table 3.3** representative of this list.

Table 3.3 *Tree excluded compounds from the 35 present in the "excluded" library in* ***Appendix II***.

Chemical excluded	Description	Toxicological information [21]
Corticosterone	Present in all blood samples, including in blind blood.	Endogenous steroid hormone. Non-PTRC.
Cotinine	Present almost all blood samples, including in blind blood.	Alkaloid metabolite of Nicotine. Rarely toxic.
Tryptophan	Present in all injections, including in blind blood.	Endogenous amino acid. Non-PTRC.

Summarizing, 35 compounds from the targeted library were included in the "excluded" list of compound. These results were of importance for the filter development study.

3.1.5 Software filters

In the method evaluation context, while undergoing targeted screening analysis, two additional filters were developed, with the objective of reducing the FP rate verified while using the software´s standard filter. Hence, 3 filters were accounted for in the targeted screening stage of this STA: "Identified" filter, Filter I and "Adduct" filter.

"Identified" filter

The parameters are present in the topic **"Filters"** of the **section 3.1.1**. As stated, this filter´s criteria were very broad and produced a great amount of FPs.

Filter development

To address the FP rate problem a filtering study was delineated. The filter study was a possibility because of the export option of the software, which enabled the export of the information exclusively displayed in the "Component Summary", present in the "Analysis Method" window. An example is displayed in **Figure 3.5** for the injection relative to sample TIM2938. There, in the "View" denominated "EXPORT View" it was stored the information that was wanted to be exported. The information displayed included: component name, molecular formula, identification status, analytically acquired RT, response of the hit, and the presence of adducts.

Component Summary ▾ View: *EXPORT View

	Component name	Formula	Identification status	Observed RT (min)	Response	Adducts
1	2-Ethylamino-1-(3,4-Methylenedioxyphenyl)propan-1...	C12H15NO3	Identified	2.25	209	+Na
2	Acefylline	C9H10N4O4	Identified	1.47	120	+Na
3	Ajmaline	C20H26N2O2	Identified	4.13	251	+K
4	Articaine	C13H20N2O3S	Identified	3.94	246	+K
5	Celiprolol	C20H33N3O4	Identified	4.45	3635	+K
6	Clenbuterol	C12H18Cl2N2O	Identified	3.94	4680	+H
7	Codeine	C18H21NO3	Identified	1.76	31195	+H, +K, +Na
8	Codeine, Glucuronide	C24H29NO9	Identified	1.29	8106	+H, +K, +Na
9	Codeine, Nor	C17H19NO3	Identified	1.62	358	+H, +K, +Na
10	Doxepine	C19H21NO	Identified	6.99	245	+H

Figure 3.5 *"Component Summary" of the "Analysis Method" of sample TIM2938, which displayed exclusively data selected in the "EXPORT view".*

The file was exported in *Excel*™ format (displayed in **Table 3.4**), which reflected the order of the columns of the "Component Summary" present in **Figure 3.5**, with three additional columns that facilitated the filter study.

Table 3.4 *Excel format of the UNIFI1.7™ exported data. Additional column* ***(I)*** *for conjugation of all injections into one spreadsheet.* ***(II)*** *Additional column created for classification purposes* ***(III)*** *column to display utilized filter in the analysis.*

	Injection_Name	Component_name	Formula	Identification status	Observed RT (min)	Responce	Adducts	Label	Filter
1	TIM2938	2-Ethylamino-1-(3,4-Methylenedio	C12H15NO3	Identified	2,25	209	+Na	FP	"Identified"
2	TIM2938	Acefylline	C9H10N4O4	Identified	1,47	120	+Na	FP	"Identified"
3	TIM2938	Ajmaline	C20H26N2O2	Identified	4,13	251	+K	FP	"Identified"
4	TIM2938	Articaine	C13H20N2O3S	Identified	3,94	246	+K	FP	"Identified"
5	TIM2938	Celiprolol	C20H33N3O4	Identified	4,45	3635	+K	FP	"Identified"
6	TIM2938	Clenbuterol	C12H18Cl2N2O	Identified	3,94	4680	+H	FP	"Identified"
7	TIM2938	Codeine	C18H21NO3	Identified	1,76	31195	+H, +K, +Na	UP	"Identified"
8	TIM2938	Codeine, Glucuronide	C24H29NO9	Identified	1,29	8106	+H, +K, +Na	UP	"Identified"
9	TIM2938	Codeine, Nor	C17H19NO3	Identified	1,62	358	+H, +K, +Na	FP	"Identified"
10	TIM2938	Doxepine	C19H21NO	Identified	6,99	245	+H	FP	"Identified"
	(I)							**(II)**	**(III)**

The 55 samples analyzed in this project were distributed through 6 batches. Each batch had injections that were classified as forensic case sample (TIM followed by four algorisms) and quality control injection (e.g. BAMBS). As depicted in the "Summary Plot" in **Figure 1.26**, an analyte listed in the targeted library will give a detector response (= or ≠ 0) in every injection. However, when exporting the *UNIFI1.7*™ data to *Excel*™ format, each injection will produce results displayed in separated spreadsheets. To facilitate the analysis, a *macro* was programed in order to put, for each one of the 6 batches, every injections´ results into a singular spreadsheet. Hence, column (**I**) presented in **Table 3.4**, denominated "Injection_Name", listed every injection which would be otherwise separated.

Column (**II**) is supplementary to what was exported by the software. It contained classification of the various hits, which was indispensable for the filter development. The column was purposely named "Label" so *the* software could recognize the data when imported back from *Excel*™ format data to *UNIFI1.7*™ format. This way, for every batch, it was possible to display a "Label" column in the "Summary Component" which provided immediate classification of any analyte in the "Component Summary".

Column (**III**), named "Filter" was added to the exported *Excel*™ file so it could be possible to compare the FP rate reduction capabilities of each filter created.

Filter I

Following the procedure previously described, theoretical filter development was done based on *Excel*™, so the parameters for a software filter could be acquired. The objective was to discover a filter that reduced the FP rate to a minimum without influencing the sensitivity of the method, *i.e.* the amount of UPs and FNs.

The results of the filter development are present in **Figure 3.6**, which displays the parameter adopted in the software Filter I.

As in the case of the "Identified" filter the "Identification status" was plotted as "Identified", meaning that the screening would target only for those chemicals present in the targeted library. However, in the case of Filter I case, the information provided in the "excluded" library (**Appendix II**) was implemented in the analysis. This meant that in the 1030 target library, 35 compounds were plotted as excluded from the analysis, which dramatically contributed for the reduction of the FP rate. Hence, the field relative to "excluded" was set to "Yes".

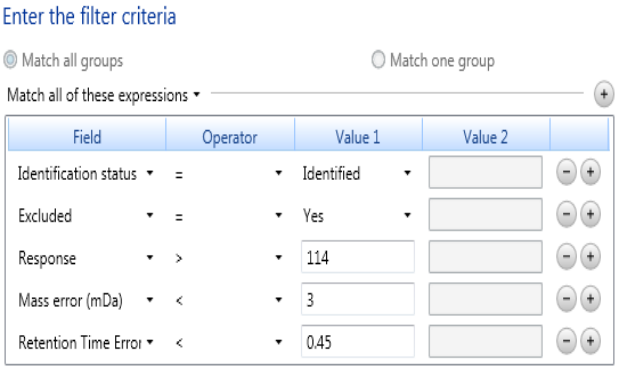

Figure 3.6 *Software filter I* set parameters.

All responses in the "Component Summary" were considered from the value 114 onwards, as terbutaline hit of sample TIM2967, from batch 2, was the UP from all the targeted screening (employing the "Identified" filter) with the lowest response (114.108). Terbutaline is a β_2-adrenergic receptor agonist and it is employed as a "reliever" inhaler in the management of asthma symptoms [21]. The hit was further classified as CP following comparison with the results listed in the LIMS database. The cause of death was directly related to a lung disease.

To additional parameters such as mass error and RT error it was attributed a maximum of 3 mDa and 0.45 min, respectively. These parameters were preserved from the "Identified" filter.

"Adducts" filter

From the results of the targeted screening, parameterized by the "Identified" filter, it was possible to attain that any hit that had spectral information of the adducts Na^+ and K^+ or both, but not of the protonated molecule were designated FP. Hence, a virtual theoretical filter was developed, excluding all hits with this particularity.

This "Adducts" filter considered the same parameters as Filter I but it had an additional field named "Adduct" that was set as "excluded". In the theoretical *Excel™* based study, this filter was the one that most rectified for the presence of FP in the results without, conditioning the sensitivity of the method.

All results from the filter study are presented and discussed in **section 3.1.7**.

3.1.6 Target analysis results for the 55 forensic samples

The overall results of targeted screening for PTRCs in the 55 forensic whole blood samples are presented in **Appendix III**. The example of case TIM2938 (see **Table 3.5**) is representative for all the analyzed samples. The screening was elaborated employing the "Identified" filter as the amount of FP was not of interest at this stage and exclusively sensitivity aspects of the developed method were of relevance.

The column titled "Case" lists all the forensic samples involved in the targeted screening analysis. "RKA Report" column states the conclusion reached by the department concerning the cause of death, in the case of a *postmortem* analysis. It was stated "*antemortem*" for cases in which the person was alive during toxicological investigation. The column "Compounds" lists all the chemicals identified either by *UNIFI1.7*^{TM} or by parallel methods employed in RKA. The column designated "*UNIFI1.7*^{TM}" lists the independently acquired results in the method developed in this project. Due to the amount of data, FP hits are absent from **Appendix III**. Exclusively the UPs and FNs are listed in the column.

Table 3.5 *Target screening results of sample TIM2938, present in* ***Appendix III.***

Case	RKA report	Compound	*UNIFI1.7*^{TM}	LIMS (mg/Kg)	FN evaluation
TIM2938	Kidney disease	Levomepromazine	UP	0.024	
		Enalapril	**FN**	0.001	LTC Above
		Hydrocodone	UP	Negative	
		Codeine	UP	0.16	
		Codeine, glucuronide-	UP	Negative	
		Morphine	**FN**	0.04	**LTC Below**

In the column titled "LIMS (mg/Kg)" it is shown the concentration at which the chemical was identified. The column presents "negative" results for compounds found by the method hereby developed, but missed in every parallel instrument employed in the investigation of the same forensic case.

Lastly, the column "FN evaluation" assesses the relevance of compounds absent in the *UNIFI1.7*^{TM} analysis. The evaluation was done, with regard to the LTC (listed in **Appendix I**) for each FN. If the measured concentration, listed in LIMS database, surpassed the LTC, then the FN was considered toxicological relevant for analysis. In the eventuality of a measured concentration being below the respective LTC, the FN was considered redundant as it had no influence in the toxicological diagnostic. In the case of FNs with LTCs

below the measured concentration, additional parameters were accounted for in their relevancy evaluation.

Hence, if in the sample it was detected, by *UNIFI1.7™*, the parent drug or a metabolite of the compound absent in the screening developed in this thesis, the FN was considered redundant. Additionally, if in the same sample it was identified a compound (UP) with the same toxicological properties as the FN, the latter was considered redundant (see the example, in sample TIM2955, of morphine and 6-MAM, present in **Table 3.2**, page 59). Furthermore, if the FN was considered a non-PTRC it was considered redundant for diagnostic. Such was the case of ibuprofen (at 5 mg/Kg) in sample TIM3027, as it is an over-the-counter non-steroidal anti-inflammatory drug (NSAID) [21]. Additionally, this compound is only detectable in negative ionization mode, which wasn´t covered by this project.

In the *postmortem* forensic case exemplified in **Table 3.5**, it was registered 2 FNs. Enalapril, which LTC is higher than the concentration provided in LIMS, so it was considered irrelevant for cause of death appointment. The other FN was morphine which LTC is 4 times lower than the concentration provided by the LIMS database. As the deceased hadn´t a history of drug of abuse consumption, the morphine present in the sample was probably an active component in a pharmaceutical administered to the patient, diagnosed with a kidney disease.

Morphine is the gold standard in commercialized analgesics [23], contrarily to 6-MAM. As there wasn´t heroin intake, the 6-MAM wasn´t present in the blood sample and so this FN was one of the two hits that were evaluated as important misses (see **Table 3.6**) by the method herby developed.

Table 3.6 *Targeted screening statistical results*

Classification	No. of hits	No. of compounds
UP	231	103
CP	119	53
LIMS negative	112	58
Total FNs	28	18
Redundant FNs	26	17
Important FNs	2	1

The table was assembled based on the data interpretation parameters previously discussed and on the results provided in **Appendix III.**

From **Table 3.6**, an analysis of the sensitivity of the method´s targeted screening was possible (see **Table 3.7**) and it was based on the **equation (1.10)** present in **section 1.5.3**.

Table 3.7 *Target screening sensitivity in function of FN evaluation*

FN	Sensitivity (%)
Total	$\frac{231}{(231+28)} \text{x } 100 = 89\,\%$
Important	$\frac{231}{(231+2)} \text{x } 100 = 99\,\%$

Hence, from a holistic point of view, the sensitivity of the method was of 89 %. After the toxicological evaluation of the 28 hits classified as FN, the sensitivity raised to 99 %.

Despite an increase to an almost flawless sensitivity, 2 FNs didn´t permit the method to be in agreement to Roman *et. al.* [71] condition, which states that a targeted screening, in a STA, should provide a sensitivity of 100 % [71].

The two FNs corresponded to morphine hits in samples TIM2938 and TIM3012. In both cases the compound was present in a concentration several times above its LTC and 6-MAM or other metabolite of heroin were not targeted identified.

This compound is the most problematic to be identified and, in the case of TIM3012, the compound wasn´t possible to be detected by the instrument. Hence, it can be concluded that this method is somewhat ineffective to screen for morphine. Another method has to be run in parallel to rectify this instrument/software limitation.

Additionally, a pertinent FN was phenobarbital in sample TIM3052. As a barbiturate, it is a neutral but fairly polar compound, however its analysis is confined to methods employing negative ionization in the ion source [69]. This PTRC was the cause of death in this forensic case but its absence in *UNIFI1.7™* wasn´t classified as an important FN, due to the fact that this method did not listed the compound in the library. Additionally, even integrated in the suspect library for semi-targeted screening, it wasn´t able to be identified. This is justified with the fact that this 3 staged screening method was exclusive for basic chemicals and the ionization polarity in the source was set to positive in all stages of the analysis.

To assess the sensitivity of this method to barbiturates, a run of the sample TIM3052 should be done, in the same instrument, but in ESI negative mode [69].

As it wasn´t possible to perform this procedure, this FN hit was classified as inconclusive.

Despite some drawbacks, this method was able to obtain a generous amount of hits classified as UP but absent in the LIMS database. Indeed, for the 55 samples analyzed, 112 hits were exclusively identified by the method developed in this project, but not by any of the methods ran in parallel.

3.1.7 False positive frequency

It is stipulated that an STA has to be time efficient and sensitive [71]. As a sensitivity of 100 % wasn´t possible, for the reasons stated in the previous section, a specificity study was implemented in order to provide the lowest number of FP hits without influencing the UP hits obtained in the analysis parameterized by the "Identified" filter.

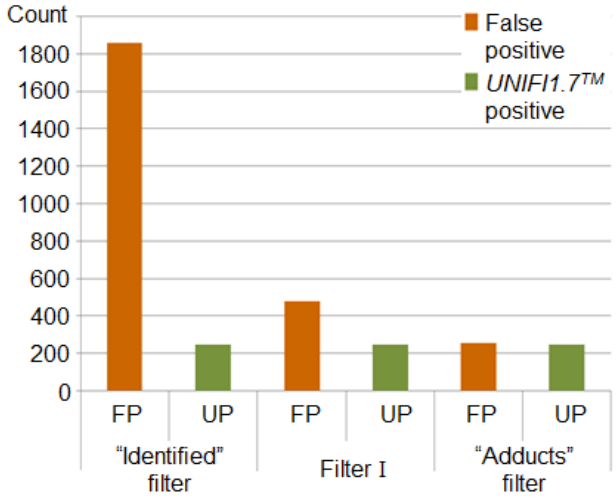

Figure 3.7 *Filter comparison regarding FP and UP hit count.*

A comparison between the three filters considered in **section 3.1.5** regarding the number of FP and UP hits was performed and the results are presented in **Figure 3.7**.

From the graphical information, it is possible to attain all that the filters provided the same amount of UP, hence there wasn´t variability between the sensitivity of the method during the filter study. The only aspect that varied from the filter with the broadest parameters ("Identified" filter), to the one with the narrower filter criteria ("Adducts" filter), was the amount of hits classified as FPs. In **Table 3.8** specific quantities and the FP frequency (see **equation 1.9** in **section 1.5.3**) for each filter are listed.

Table 3.8 *FP and UP count, and FP rate.*

Filter	No. de FP	No. de UP	Frequência de FP (%)
"Identified" filter	1859	231	$\frac{1859}{(1859+231)} \mathrm{X}\, 100 = 89$
Filter I	479	231	$\frac{479}{(479+231)} \mathrm{X}\, 100 = 68$
"Adducts" filter	256	231	$\frac{256}{(256+231)} \mathrm{X}\, 100 = 53$

In Roman *et al* [71] it was developed a targeted screening method with a targeted library containing 240 PTRCs. Based on that amount, the author defined that the number of FP should be 5 % of the total hits in each analysis. Therefore, as the developed method employed a targeted library containing 1030 compounds, a FP frequency of 22 % was considered optimal to allow a time efficient but sensible analysis.

Information about the filters´ parameters is present in **section 3.1.5**, and from **Table 3.8**, the filter that better reduced the overall number of FP without compromising the UP hits was the "Adducts" filter. However, due to a *UNIFI1.7™* deficiency, it was not possible to apply this theoretical filter as a software filter. For example, if in the "Enter the Filters Criteria" window (see, eg. **Figure 3.6**) it was selected the exclusion of hits that were exclusively

detected as a Na^+ adduct (not providing the protonated molecule in the spectrogram), the filter would exclude all hits that provided the Na^+ adduct, including those associated with the protonated ion (which presence is compulsory to classify the hit as a UP). In this way the "Adducts" filter was excluded from analysis and the problem was reported to the manufacture´s software developers.

Thereof, Filter I (criteria present in **Figure 3.6** and explained in **section 3.1.5**) was the most suited to be employed in the method targeted screening. Comparatively to the "Identified" filter, it provided a reduction of 74% in the number of FP hits without influencing the sensitivity of the method. Despite its FP frequency was 3 times greater than that proposed by Roman *et al* [71], the time required to analyze each injection varied from 2 to 20 minutes, which was considered acceptable for RKA standards.

3.2 Semi-targeted screening

3.2.1 Semi-targeted library

As referred in **section 1.4.9**, the main distinctions between targeted and the semi-targeted screenings are the fact that the latter neglects the RT of all compounds and, in most cases, it only accounts for the suspect molecular formula, which is then translated to exact mass by the *UNIFI1.7™*.

The semi-targeted database was assembled in *Excel*™ format and lists 1392 compounds of toxicological relevance obtained from 14 different sources (see **Table 3.9**). The compounds were absent in the targeted library and for each, compound name and molecular formula were compulsorily plotted in the semi-targeted library. Additional compound information present in the database was: molecular structure encrypted in a *.mol.* file; exact mass of fragments obtained from literature; IUPAC name; drug classification based on compound structure; and CAS Number.

Table 3.9 *Extract of the in-house built semi-library.*

Compound Name	Formula	Structure	Adduct	RT	Frag1	Frag2	Frag3	Frag4	IUPAC Name	Class	CAS No.
2C-E	C12H19NO2				193,1223	178,0988	135,0804		2-(4-ethyl-2,5-dimethoxy-phenyl)ethanamine	2C Family	71539-34-9
2C-H	C10H15NO2				165,091				2-(2,5-dimethoxyphenyl)ethanamine	2C Family	3600-86-0
2C-I	C10H14INO2				290,9876				2-(4-iodo-2,5-dimethoxyphenyl)ethanamine	2C Family	69587-11-7
3-4-DMA	C11H17NO2				151,0754	179,1067	121,0648		1-(3,4-dimethoxyphenyl)propan-2-amine	Amphetamines	120-26-3
4-FA	C9H12NF				109,0448	137,0761			1-(4-Fluorophenyl)propan-2-amine	Phenethylamines	459-02-9
4-FMA	C10H14NF				109,0448	137,0761			1-(4-Fluorophenyl)-N-methylpropan-2-amine	Phenethylamines	351-03-1
BDB	C11H15NO2				135,0441	177,0910			1-(1,3-Benzodioxol-5-yl)butan-2-amine	Phenethylamines	42542-07-4
DPT	C16H24N2				144,0808	114,1277	118,0651		N-[2-(1H-indol-3-yl)ethyl]-N-propylpropan-1-amine	Tryptamines	61-52-9
Ethylcathinone	C11H15NO				105,0699				(RS)-2-ethylamino-1-phenyl-propan-1-one	Cathinones	51553-17-4
Harmaline	C13H14N2O				174,0913	200,0944	131,0729		7-methoxy-1-methyl-4,9-dihydro-3*H*-pyrido[3,4-*b*]indole	Plants	304-21-2
Harmine	C13H12N2O				198,0788	170,0838			7-MeO-1-Me-9H-pyrido[3,4-b]-indole	Plants	442-51-3
MDDMA	C12H17NO2				135,0441	163,0754	133,0648		1-(1,3-benzodioxol-5-yl)-N,N-dimethylpropan-2-amine	Amphetamines	131206-60-5
Methedrone	C11H15NO2				135,0804	105,0699			1-(4-methoxyphenyl)-2-(methylamino)propan-1-one	Cathinones	530-54-1
Methoxyphenamine	C11H17NO				121,0648	91,0542	149,0961		N-methyl-1-(2-methoxyphenyl)propan-2-amine	Phenethylamines	93-30-1
Normethadone	C20H25NO				251,1430	223,1117	195,1168		6-(Dimethylamino)-4,4-diphenylhexan-3-one	Opioids/Opiates	467-85-6
Normorphine	C16H17NO3				229,0859				(5α,6α)-7,8-Didehydro-4,5-epoxymorphinan-3,6-diol	Opioids/Opiates	466-97-7
Pethidine	C15H21NO2				220,1332	174,1277	202,1226		1-Methyl-4-phenyl-isonipecot ethyl ester	Opioids/Opiates	57-42-1
Phenmetrazine	C11H15NO				134,0964	117,0699	91,0542		3-Methyl-2-phenylmorpholine	Phenethylamines	134-49-6
Phenylpropanolamine	C9H13NO				134,0964	119,0729			2-amino-1-phenylpropan-1-ol	Amphetamines	14838-15-4
Tiagabine	C20H25NO2S2				247,0610	278,1209	149,0419		(R)-1-[4,4-bis(3-methylthiophen-2-yl)but-3-enyl] piperidine-3-carboxylic acid	Pharms	115103-54-3
Tilidine	C17H23NO2				155,0855	129,0699	119,0855		2-(Dimethylamino)-1-phenyl-cyclohex-3-ene-1-carboxylic acid ethyl ester	Opioids/Opiates	20380-58-9

The different sources used for database assembling can be divided into websites, scientific literature, RKA research, international organizations and drug forums.

Websites

- *Southern Association of Forensic Scientists*. This site displays a database providing molecular formula and structure of common and uncommon drugs of abuse. Its elaboration is the result of the crossing of analytical results from seized drugs in an inter-laboratory strategy. Its main purpose is to serve as an analytical tool in order to facilitate the identification of unknowns [116]. It contributed with 357 compounds for the semi-targeted library.
- *Wikipedia*. A free-access/collaboratively edited Internet encyclopedia in which the listing of "designer drug" is actively actualized [117]. It contributed with 38 suspect compounds.
- *Chemograph Plus*. This website corresponds to a German language program for formulation of chemical structures based on adulterations of previously known molecular structures. It´s main purpose is the provision of predictions of the black market manufacturers´ future productions [118]. It provided 325 suspect compounds to the library.
- *Chemicalsoft*. This software provided a mass spectra library of reference compounds fragmented at an energy ranging from 20, 35 and 50 eV. It has a specific section for "designer drugs". It provides nominal mass of parent and product ions [119]. It contribuited with 88 compounds to the library.

Scientific literature

- de Castro, *et al*. [120].This research covered a validation of a targeted-screening method based on AM acquisition. It provided the backbone for the semi-targeted library developed in this project. This paper supplied the suspect database with exact mass of product ions, predicted with resource to ACD/MS Fragmenter™. 145 suspect compounds with the respective fragment(s) exact mass at a collision energy of 40 eV, were added to the developed database [120].
- Roman et.al. [71]. This paper revealed a similar procedure to that developed in de Castro, et. al [120]. 27 additional common drugs of abuse and pharmaceuticals, absent in the hereby utilized targeted library, were added to the suspect library.

On-going research

- PhD research taking place in RKA provided additional 154 compounds relative to synthetic cannabinoids. It provided the parent drug and metabolites exact masses, including those of the respective product ions.

International organizations

- *EMCDDA*. The data presented in this organization's website provided a table outlining the similarities and differences of the national system in the EU and Norway, regarding the classification of drugs and precursors according the three UN Conventions of 1961, 1971 and 1998 [121]. The report provided additional 10 suspect compounds.
- *UNODC:* The website of the United Nations Office on Drugs and Crime provided a report highlighting the importance and the challenges behind the identification of new psychoactive substances [122]. Additional 173 compounds with the respective structures were added to the semi-targeted library.

Drug forums

- *Erowid Experience Vaults*. Website where it is attempted to catalog a wide variety of experiences people have with psychoactive plants and chemicals [123]. 60 compounds absent in the targeted library where found in the testimonial reports and added to the semi-targeted library.
- *Drug-Forum. This website is similar to* Erowid [124]. The database there presented contributed with 15 compounds for the suspect library assembling.

3.2.2 Sample analysis based on import of fragment´s AM

From the total amount of compounds present in the semi-targeted library, 352 had at least one fragment with exact mass. All product ions' AMs were provided by literature [71, 120].

Compound fragmentation pattern information can be imported by plotting the exact mass of the fragments in the convenient slot.

Table 3.10 exemplifies an importation template for 5 analytes to which it was possible to obtain exact mass values for the fragments.

Table 3.10 *Importation table for 5 analytes containing fragments with exact mass.*

Analyte	Formula	Structure	Adduct	RT(min)	F1	F2	F3	F4
2C-I	C10H14INO2			0.5	290.988			
2C-D	C11H17NO2			0.5	164.107	149.084		
2C-E	C12H19NO2			0.5	193.122	178.099	135.082	
2,5-Dimethoxy-4-(n)-propylthiophenenthylamine	C13H21NO2S			0.5	239.110	197.063	182.045	
NMT	C11H14N2			0.5	143.073	158.096	132.081	

The *Excel*™ table has to be always in this format, even if there were empty columns, such as "Structure" and "Adduct" in this case.

Additionally, it is important to state that the table contains a value for RT, even though this criterion isn't of relevancy for the semi-targeted screening. This happens because, in

every occasion, *UNIFI1.7™* has to detect a RT value in order to import a library. This value (0.5 min in this case) can be deleted afterwards, in the software settings.

The same 6 batches used in the targeted screening were then updated in order to delete the targeted-library and import the semi-library with 1392 compounds.

As with the targeted library, all the compounds in the in-house database were now regarded as "identified", which is important for filtering purposes.

A new Filter II was developed to meet the required criteria for a time efficient semi-targeted screening and is displayed in **Figure 3.8**. The parameters there present were the ones that were considered for every stage of the semi-targeted screening, of the same 55 whole blood samples treated in the targeted analysis.

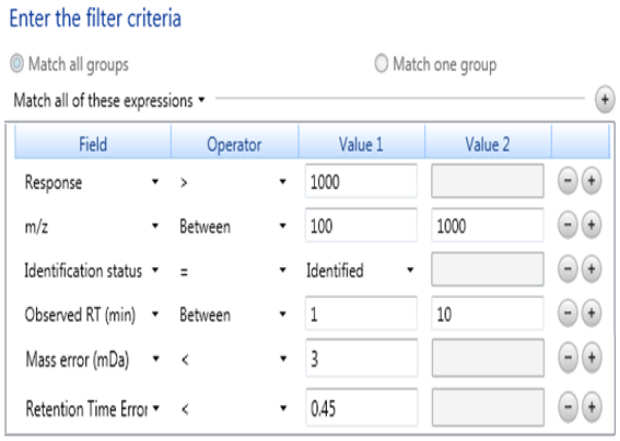

Figure 3.8 *Software Filter II parameters.*

Only compounds with responses above 1000 were to be taken into account. Given the amount of FPs in the targeted screening with a response threshold of 114, it was concluded that for semi-target analysis a higher threshold had to be used, as the screening considered a larger library and produced more hits due to isomerism. The m/z was selected to be below 1000 as it is screened for small molecules. The identification status was "Identified", like in Filter I (**Figure 3.6**), but now with a different compound library.

The considered RT was less than 10 min, so all the compounds that eluted after this time were filtered out, as it was previously observed that they corresponded to interferences.

Mass error (mDa) and RT error were preserved from the filter used in targeted screening.

The first stage of the semi-targeted analysis of the 55 whole blood samples, comprehended the import of the 1392 compound semi-library, with 352 compounds with predicted fragments with exact mass, provided by literature. All fragmentation information from these suspects was exclusively imported as exemplified in the **Table 3.10**.

At this stage of semi-targeted screening, based on prediction of exact mass of fragments by softwares other than *UNIFI1.7™*, 7 hits ware considered UP and 6 compounds were identified. Results are presented in **section 3.2.5**.

Among the semi-targeted identifications, there is ritalinic acid in sample TIM3052, illustrated in **Figure 3.9**. This example of semi-targeted screening provided complimentary information to that obtained in the targeted analysis of the same sample (see **Figure 3.1**).

In **Figure 3.9 (I),** it is presented the TIC where 2 major hits, labeled as pirbuterol (1.05 min) and lysergic acid amide (2.73 min) where later classified as FP. Ritalinic acid, the inactive major metabolite of methylphenidate [125], was not visible as it had a peak with a comparative lower intensity.

Subfigure **(II)** is relative to the XIC for the exact mass of 220.13325 m/z, which is exclusively characteristic to ritalinic acid (2.83 min). In this case there is only one hit for the suspect in analysis. This is a plausible occurrence when not providing RT in the library, but generally, it is common to exist, for a single suspect analyte, multiple hits in the XIC. This is dependent in the number of isomers in the injection (which is zero in this example). This matter will be further discussed in **section 3.2.4**.

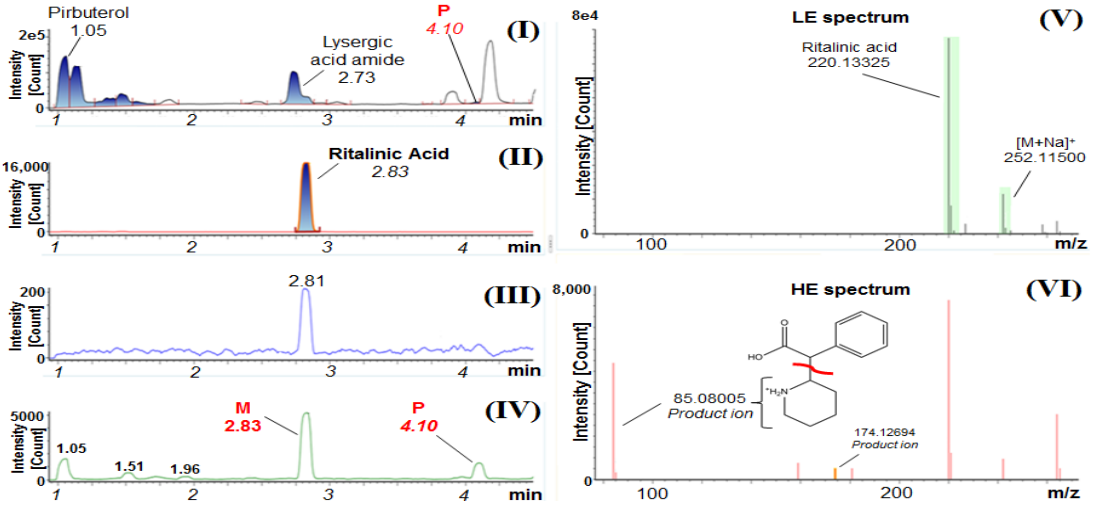

Figure 3.9 *UNIFI1.7™ "Analysis Method" window of detection for ritalinic acid in sample TIM3052.* **(I)** *TIC evidencing two suspect hits with high intensities and a discreet non-identified peak at 4.10 min corresponding to compound P;* **(II)** *XIC for the mass 220.13325 m/z, which characterizes the protonated molecule of ritalinic acid;.* **(III)** *XIC for the mass 174.12694 m/z, exclusively related to the identified product ion;* **(IV)** XIC for the mass 85.08005 m/z, *characteristic of the fragment produced both by suspect M (2.83 min) and compound P (4.10 min);* **(V)** *Low collision energy spectrum for the elution time of 2.83 min;* **(VI)** *High collision energy spectrum for the chromatographic peak at 2.83 min.*

In the second XIC (subfigure **(III)**, relative to the mass 174.12694 m/z, a fragment was identified but can be classified as irrelevant as the intensity (just above 200 counts) is too low for a clear correlation with other PTRCs in the sample.

The XIC **(IV)**, of the mass 85.08005 m/z, is of relevance in terms of fragmentation pattern information, as it correlates ritalinic acid with other compounds present in the same sample, such as those that eluted at 1.05, 1.51, 1.72, 2.44 and 4.20 minutes. The latter is the RT of compound "P", correspondent to methylphenidate which was identified in the targeted analysis, (illustrated in **Figure 3.1**), and ritalinic acid was the compound "M" of the same figure, but was now possible to be identified as it was present in the suspect library.

The provision of the fragment with mass of 85.08005 m/z made possible a correlation between a parent compound (methylphenidate) and its TP (ritalinic acid). This satisfied the identification criterion which states that the presence of a metabolite (even if not a non-PTRC) enhances the credibility of the precursor ion´s hit.

The spectra provided in the right quadrant of the **Figure 3.9** reveal the suspect´s protonated ion (220.13325 m/z) and its sodium adduct (252.11500 m/z) in the LE spectrum (**Figure 3.1 (V)**). Additionally, the already referred fragments (174.12694 m/z and 85.08005 m/z) are presented in the HE spectrum (**Figure 3.1 (VI)**). The ritalinic acid molecular structure illustrated was not directly provided by the software, as this is not the case of a semi-targeted screening based on fragmentation prediction acquired from molecular structure importation. The structure is exclusively presented for elucidation reasons.

3.2.3 Validation of fragment prediction based on molecular structure import

The previously discussed hit was possible after importation of fragmentation exact mass predicted by literature. However, it was found that *UNIFI1.7™* enclosed the capability of predicting, by itself, fragmentation. Literature revealed that *ACD/MS Fragmenter*™ is the tool of choice for product ion prediction [71, 120].

As there isn´t any literature disclosing *UNIFI1.7™* capabilities in this ambit, this project firstly provided an evaluation study before implementing this software´s feature in the method hereby developed.

The experiment comprehended 4 designer drugs:

- Pyrovalerone: a pyrrolidinophenone based designer-drug;
- 5-FUR-144 (or XLR11): a tryptamine based designer-drug;
- 5-F-AKB48: a tryptamine based designer-drug;
- α-PVP: a pyrrolidinophenone based designer-drug.

All 4 compounds were not present in the targeted library, but they were listed in the suspect-library. Pure standards were available *a priori*.

This experiment had the objective of, with a narrow suspect-library, evaluate if it was possible to detect and identify the 4 compounds diluted in whole blood and under controlled conditions with both methods of fragmentation prediction: *AC/D MS Fragmenter™* and *UNIFI1.7™*.

The procedure was divided in 3 parts: spiking, fragmentation prediction by *UNIFI1.7™* and correlation with *AC/D MS Fragmenter™* fragment prediction.

3.2.3.1 *Blood spiking with 4 pure standards*

- The respective pure standard solutions of all 4 compounds, at a concentration of 1.0 mg/mL were diluted in a aqueous solution of MeOH (50:50 v/v) to make the respective 4 solution at a concentration of 1 µg/mL;
- A volume of 2 µL of each resulting solutions was spiked into three blind blood samples with volumes of 100 µL, in order to produce three homologous biological matrixes with a representative concentration of 20 ng/mg each;
- Each tube was shacked and undergone further automatic sample preparation (see procedure in **section 2.3.1**);
- The three samples were then injected into the instrument;
- The resulted raw data was analyzed through *UNIFI1.7™;*
- The software filter employed in the study was the "Identified" filter (parameters presented in **section 3.1.1**).

3.2.3.2 Import of molecular structure to *UNIFI1.7™*

To exemplify the import procedure of molecular structure for prediction of fragmentation of intended analytes, **Table 3.11**, provides the importation of the 4 designer drugs used for validation of the *UNIFI1.7™* capability of predicting the fragmentation pattern of suspects.

Table 3.11 *Import table for 4 designer drugs containing molecular structure for further fragmentation prediction.*

Analyte	Formula	Structure	Adduct	RT(min)	F1	F2	F3	F4
α-PVP	C15H21NO	α-PVP		0.5				
Pyrovalerone	C16H23NO	Pyrovalerone		0.5				
5F-AKB48	C23H30FN3O	5F-AKB48		0.5				
XLR11	C21H28FNO	XLR11		0.5				

To obtain the structures it was drawn in the *ChemBioDraw Ultra 12.0™* the respective molecular structures in *.mol* format.

The RT corresponds to 0.5 min for the same reason explained in **section 3.2.2**.

It is possible to attain that, in this case the columns relative to fragmentation exact mass are empty and the column "Structure" has the same identification name of the *.mol* file. Both names have to be equal for correct structure importation (see **Figure 3.10**).

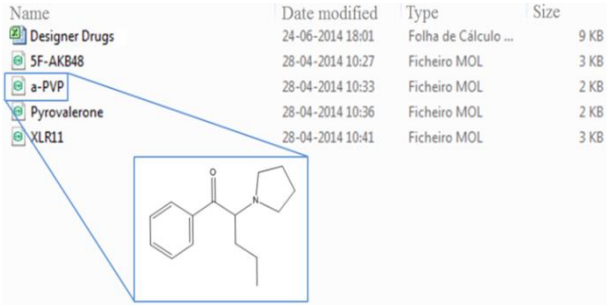

Figure 3.10 *File containing Excel™ file and .mol files*

Additionally, the *Excel*™ file that holds the import table has to be in the same folter as the respective *.mol* files when the spreadsheet is imported to the software.

The three homologous blood samples were analyzed by *UNIFI1.7*™. It is given the case of α-PVP in **Figure 3.11**, as a representative example of the analysis of the fragmentation prediction by molecular structure import.

Subfigure **(I)** is referent to the TIC of one of the homologous blood samples and it presents four peaks of high intensity: pyrovalerone (5.84 min), XLR11 (11.92 min), 5F-AKB48 (12.22 min) and α-PVP (4.42 min), the reference standard in analysis.

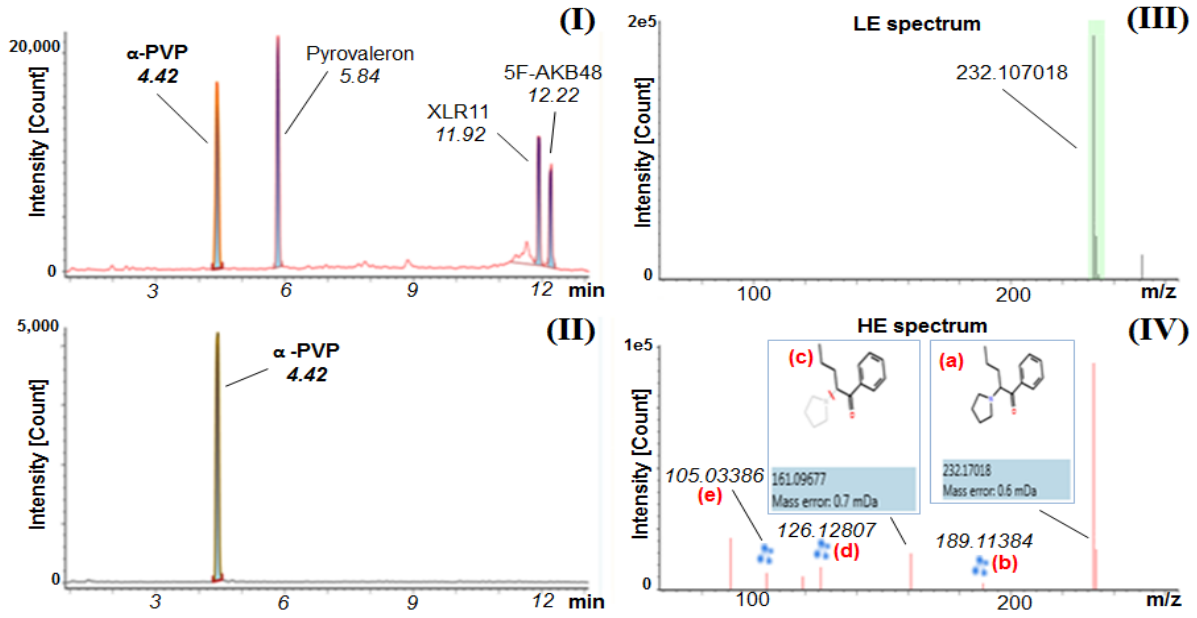

Figure 3.11 *UNIFI1.7*™ *"Analysis Method" window of detection for α-PVP in a whole blood at 20 ng/mg.* **(I)** *TIC evidencing the four spiked reference standards;* **(II)** *XIC for the mass 232.107018 m/z, correspondent to the protonated α-PVP;* **(III)** *Low collision energy spectrum for the elution time of 4.42 min;* **(IV)** *High collision energy spectrum for the chromatographic peak at 4.42 min, displaying the molecular ion (a), and its characteristic fragments (b-e).*

XIC **(II)** for the exact mass 232.107018 m/z, which is exclusive to α-PVP, presents only one peak, meaning that there is not any endogenous compound in blood that is an isomer of α-PVP, otherwise the XIC would have more than one hit for this reference standard. This could be possible as there isn´t any RT stored in the 4 compound library used in this analysis.

Figure 3.11 (III) plots the low collision energy spectrum for the elution at 4.42 min. It highlights the protonated α-PVP peak, corresponding to the mass of 232.107018 m/z.

Figure 3.11 (IV) presents the high energy spectrum of the compound eluting at min 4.42, signaled as (a) and its product ions: (b) with mass of 198.11384 m/z, (c) with mass of 161.09677 (m/z), (d) with mass of 126.12807 m/z, and (e) with mass of 105.03386 m/z. A molecular structure is illustrated exclusively for fragment (c), for illustrative convenience, as the software can illustrate the molecular structure for every fragment it predicts.

Component Summary ▾

	Component name	Identified High Energy Fragments	Observed RT (min)
1	α-PVP	5	4.44
2	Pyrovalerone	5	5.84
3	5F-AKB48	6	12.20
4	XLR11	5	11.93

Figure 3.12 *"Component Summary" of the 4 reference standard compounds.*

Using this method of fragmentation pattern prediction, *UNIFI1.7™* erroneously considers the molecular ions (parent ion (a)) as an "Identified High Energy Fragment" as it is possible to attain from the "Component Summary" of α-PVP´s "Analysis Method" (see **Figure 3.12**). This pyrrolidinophenone provides 4 fragments (b to e) and not 5, as it was previously stated in the **Figure 3.11**.

Additionally, this method of fragmentation prediction did not display any additional XIC besides the one relative to the exact mass of the protonated molecule, as it is observable in **Figure 3.11**. This is a disadvantage of the product ion prediction by part of *UNIFI1.7™*. Despite predicting 4 fragments in the case of α-PVP, the software doesn´t displays the respective XICs, as it occurs in the case of fragment prediction based on AM import.

The proof of concept of this method of product ion prediction can only be achieved with the comparison of its results with those of a homologous product ion prediction by part of *AC/D Mass Fragmenter™*.

3.2.3.3 Prediction of fragmentation by AC/D *Mass Fragmenter™*

Fragment prediction *UNIFI1.7™* results for α-PVP were compared with those obtained employing *AC/D Mass Fragmenter™*. The used parameters were the same as those stipulated in **section 2.4.2**.

The results of this software are presented in **Figure 3.13**, being α-PVP representative for the three remaining designer drugs involved in this study.

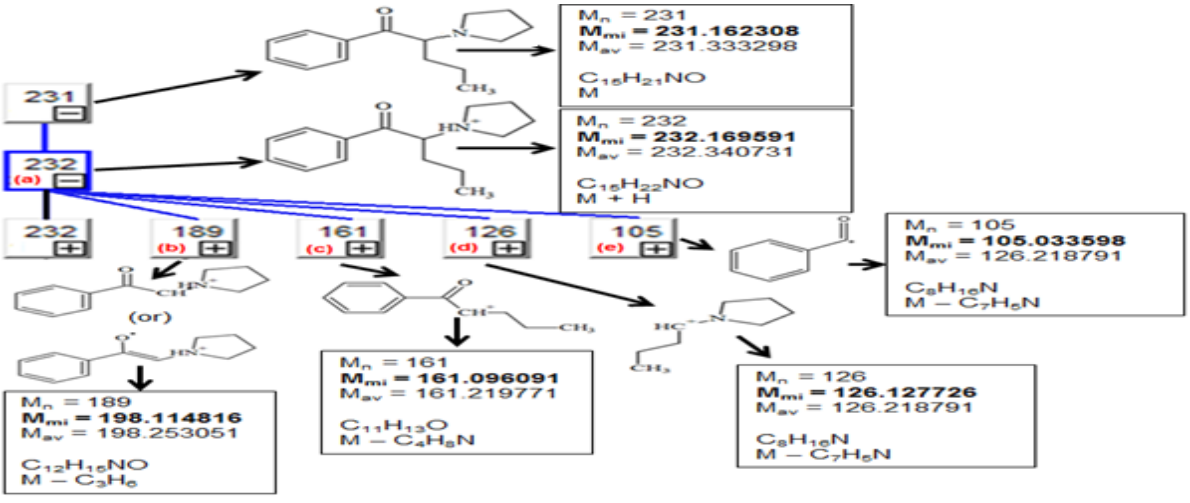

Figure 3.13 *AC/D Mass Fragmenter™ results of fragment prediction of α-PVP.*

The software displayed a tree diagram for each analyte, and each box listed information such as nominal mass (M_n), monoisotopic mass (M_{mi}), isotopes average mass (M_{av}), the chemical formula of the molecule/ion and the atoms that were subtracted from the parent compound´s molecule (α-PVP in this case). The mass of interest (M_{mi}) is highlighted for comparison purposes.

Figure 3.13 provides additional letters (a-e) as they are directly correlated with the "Identified High Energy Fragments" predicted by *UNIFI1.7™* (see **Figure 3.11 (IV)**). Indeed, it was possible to attain that, within the allowed 3 mDa deviation, all 4 fragments and the protonated molecule of α-PVP predicted in the previous method are also predicted by *AC/D Mass Fragmenter™*. The fragments possess similar monoisotopic mass. For example, in **Figure 3.11**, the product ion (c) has an exact mass of 161.09667 m/z, which has a divergence of just 0.000679 Da from that registered by *AC/D Mass Fragmenter™*.

From this it is possible to say that *UNIFI1.7™* has a similar capability of predicting the fragmentation pattern of protonated molecular ions.

However, some differences are presented in the *AC/D Mass Fragmenter™* process. As it is possible to observe, there is a ⊞ symbol in each of the 5 results in the 3rd row of the tree diagram. They inform that the software predicted additional 2nd cycle fragments from the fragments there displayed. This signifies that increasing the number of fragmentation cycles in an analysis, the higher will be the amount of fragments proposed by the software.

In the case of α-PVP, which has a structure rich in σ bounds, the fragmentation is predictable. However, in compounds such as morphine (see **Figure 3.2**) it would be very problematic to choose which fragment monoisotopic mass were to be selected for characterization studies. Additionally ambiguity can increase as more fragmentation cycles are needed to predict product ions from molecules rich in π bounds, such as steroids and opiates.

Summarizing, the three staged validation study results indicated that fragmentation prediction by *UNIFI1.7™* is a simple and time efficient process that can provide the same results as *AC/D Mass Fragmenter™*.

Despite no provision of XICs for the exact mass of the fragments proposed (which are very elucidative), it was concluded that *UNIFI1.7™* could substitute *AC/D Mass Fragmenter™* in fragmentation pattern studies.

3.2.4 Sample analysis based on fragment prediction by *UNIFI1.7™*

In **section 3.2.2**, a screening employing the fragmentation pattern prediction from *AC/D Mass Fragmenter™* was implemented to 352 compounds of the 1392 semi-targeted

library. With the *UNIFI1.7™* product ion prediction assessed, a screening including the remaining 1040 compounds was elaborated.

However, at this stage the semi-targeted library only provided ID name and the molecular formula (exact mass). Despite this limitation of criteria, it was possible to narrow down the hits acquired. Hence it was selected, for further analysis, exclusively the peaks that provided an acceptable shape and particularly low mass deviation (< 1 mDa). The number of hits that were considered acceptable was 97 and the number of compounds that were plausible to be identified by the semi-targeted screening was 61. The filter used for analysis was Filter II (see criteria in **Figure 3.8**).

To each compound of this subgroup, using *ChemBioDraw Ultra 12.0™,* the respective molecular structure was drawn. The procedure of software data importation was the same as the one described in **section 3.2.3.2**.

In the final stage of the procedure, 12 hits were classified as UP based on fragmentation pattern and 9 suspects were considered as presumptively present in the samples. Results are listed in **section 3.2.5**

Among them was 7-OH-quetiapine in samples TIM2978 and TIM2982. The analysis of the latter is presented in **Figure 3.14**. This further elucidated the advantages and disadvantages of fragmentation prediction by importation of a molecular structure. As it is possible to attain in **Figure 1.4**, quetiapine (7-OH-quetiapine parent drug) has a molecular structure susceptible to fragmentation, particularly in the non-aromatic section of the structure. Hence, abundant fragmentation was expected in its metabolite.

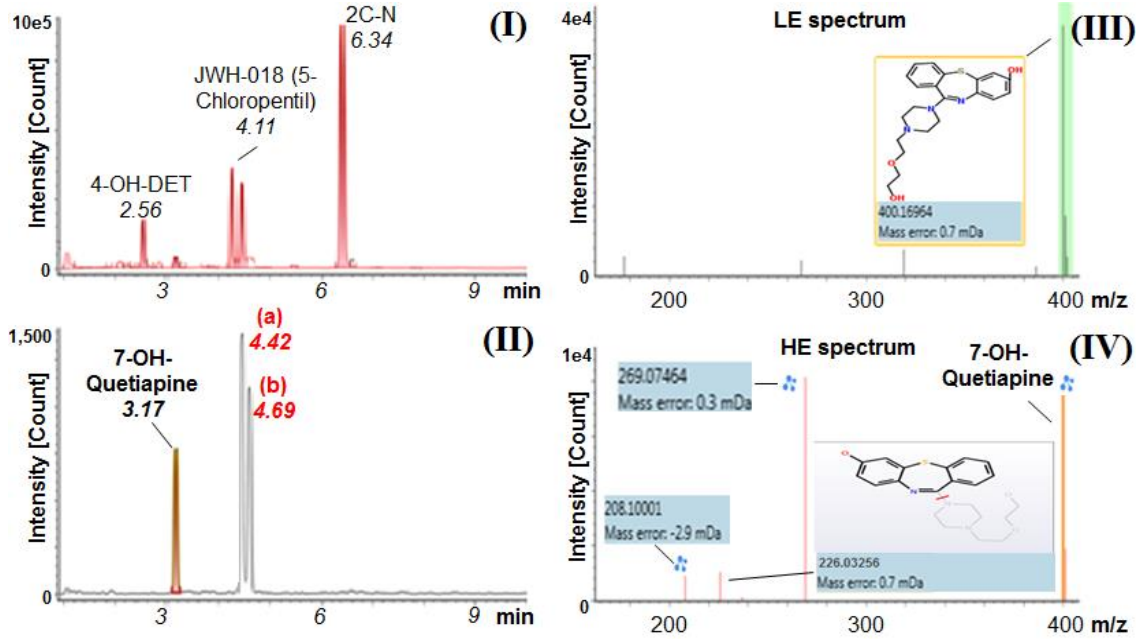

Figure 3.14 *UNIFI1.7™ "Analysis Method" window of detection for 7-OH-quetiapine in sample TIM2982.* **(I)** *TIC evidencing three suspect hits with high intensities;* **(II)** *XIC for the mass 400.16964 m/z, which characterizes the protonated molecule of 7-OH-quetiaine;* **(III)** *Low collision energy spectrum for the elution time of 3.17 min;* **(IV)** *High collision energy spectrum for the chromatographic peak at 3.17 min, evidencing 3 UNIFI1.7™ proposed fragments.*

Figure 3.14 (I) is referent to the TIC of sample TIM2982 and it presents four peaks of high intensity. Three were labeled as 4-OH-DET (2.56 min), JWH-018 (5-chloropentil) (4.11 min) and 2C-N (6.34 min). All were classified as FP.

XIC **(II)** for the exact mass 400.16964 m/z, which is relative to 7-OH-quetiapine, evidences 3 chromatographic peaks, indicating that the compound´s RT is not restrained to 3.17 min but also to 4.42 min and 4.69 min. As introduced before, suspect screening do not provide RT to the compounds listed in the library. Hence, the correlation between measured results and the data stored in the suspect library is exclusively based on the exact mass to which the measured peak corresponds and the fragment(s) that are produced in the HE spectrum. In summary, all three peaks (the hit in analysis and both peaks (a) and (b)) shared equal probability of corresponding to the correct elution time, as they provided convenient product ions in their respective HE spectra (data not provided). For this reason an additional instrumental run of the respective reference standard was compulsory to unambiguously identify 7-OH-quetiapine in the sample. Results are provided in **section 3.2.6**.

The spectra provided in the right quadrant of the figure reveal the suspect´s protonated ion (400.16964 m/z) in the LE spectrum (**Figure 3.14 (III)**). Additionally, the predicted fragments with masses 208.10001, 269.07454 and 226.03256 m/z are presented in the HE spectrum (**Figure 3.14 (VI)**). For the latter, the respective molecular structure is presented, but the same was a possibility for the remaining *UNIFI1.7™* predicted product ions.

3.2.5 Semi-targeted analysis results for 55 forensic samples

Table 3.12, provides the final semi-targeted results by conciliating both suspects semi-targeted identified, mediated by importation of fragments´ AM from literature (see **section 3.2.2**) and PTRCs identified through product ion prediction by *UNIFI1.7™* (see **section 3.2.4**).

Both product ion prediction methods could not be imported in the semi-targeted library simultaneously, as *UNIFI1.7™* prioritizes results obtained from product ion exact mass over compound structure in library, not providing the results of the latter. Hence the two analyses were effectuated separately but complementarily.

Table 3.12 *Semi-targeted screening overall results.*

Case	Semi-targeted (352+61)	Fragment prediction method	Reference standard elucidation
TIM2938	Metopon	Molecular structure import	No
	Yangonin	Molecular structure import	No
	2C-O-4	Molecular structure import	No
TIM2949	Zotepin	Molecular structure import	No
	2C-PYN BU	Molecular structure import	No
TIM2955	Zotepin	Molecular structure import	No
	Metopon	Molecular structure import	No
TIM2978	7-OH-Quetiapine	Molecular structure import	RT = 3.20 min
TIM2982	7-OH-Quetiapine	Molecular structure import	RT = 3.20 min
TIM3001	Mecloqualone	Molecular structure import	No
TIM3018	Z-10-OH-Nortriptiline	Fragment exact mass	RT = 5.78 min
	4-Ethylethcathinone	Molecular structure import	No
	E-10-OH-Amitriptiline	Fragment exact mass	No
TIM3019	Acetil-alpha-metilfentanil	Molecular structure import	No
TIM3033	E-10-OH-Nortriptiline	Fragment exact mass	RT = 4.86 min
	E-10-OH-Amitriptiline	Fragment exact mass	No
	Z-10-OH-Nortriptiline	Fragment exact mass	RT = 5.78 min
TIM3034	4-AcO-DMT	Molecular structure import	No
TIM3038	Yangonin	Molecular structure import	No
TIM3052	Ritalinic acid	Fragment exact mass	No
	Norpromazide	Fragment exact mass	No

From the table it is possible to state that 7 suspect hits were classified as UP. Their identification was based on fragment AM prediction by *ACD/MS Fragmenter*™, provided by literature [71, 120]. Among them, there are 2 *E-Z* isomers: E-10-OH-Nortriptyline and Z-10-OH-Nortriptyline. To each the pure standard was available. They were spiked in whole blood to prepare a biological mixture with a concentration of 20 ng/mg for each nortriptyline TP. The procedure was homologous to that described in **section 3.2.3**. The RTs for each diasteromer were 4.86 min for the *E* and 5.78 min for the *Z*. The retention time was coincident to those obtained in the semi-targeted screening of samples TIM3018 and TIM3033. This signified that both metabolites of the tricyclic antidepressant (TCA) [21]. were present in sample TIM3033 and the *Z* diasteromer was present in sample TIM3018. This example, besides proving the concept of semi-targeted screening based on import of fragment exact mass, it

functioned as an additional evidence to prove the presence of nortriptyline, a targeted identified PTRC in both sample TIM3033 and TIM3018.

Another identified hit based on import of AM of product ions from literature was ritalinic acid. Despite the non-availability of the respective pure standard, the information provided in both **Figure 3.1**, and **Figure 3.9**, provide important information that proves, to an acceptable extent, the presence of methylphenidate and its TP in sample TIM3052.

Additionally, 14 suspect hits were regarded as UP and the fragmentation prediction was performed by *UNIFI1.7™* after molecular structure import. Among the hits, 2 were relative to 7-OH-quetiapine (present in samples TIM2978 and TIM2982). In **section 3.2.4** the analysis of this quetiapine metabolite in sample TIM2982 was illustrated in **Figure 3.14** to demonstrate the ambiguity of RT allocation in semi-targeted screening.

In the next section it is demonstrated the elucidative capability that RT has, obtained from the pure standard, in order to prove the presence of the intended compound.

3.2.6 *UNIFI1.7™* fragment prediction proof of concept

In **section 3.2.4**, the XIC of **Figure 3.14** (**II**), page 78, provided three possibilities of elution time: 3.17 min (the analyzed peak), 4.42 min (a) and 4.69 min (b). As the reference standard of quetiapine was available, a procedure similar to that described in **section 3.2.3** was implemented. The analysis of the injection containing the spiked whole blood is displayed in **Figure 3.15**, which served as proof that 7-OH-quetiapine was present in sample TIM2982 (such as in TIM2978). Additionally it substantiates the credibility of the targeted identification of quetiapine.

Most importantly, the measurement of the RT time of 7-OH-quetiapine by injection of its pure standard and the similarity to one of the three obtained in **Figure 3.14** (**II**), proved the concept that *UNIFI1.7™* was able to perform an independent semi-targeted screening.

Figure 3.15 (**I**) displays the TIC in which only 7-OH-quetiapine was displayed, as at an intensity count of 600000 no other peak corresponding to an endogenous compound was displayable.

Figure 3.15 (**II**) displays the XIC for the mass of 400.16940 m/z, which characterizes 7-OH-quetiapine. Form the information there provided, the RT of this quetiapine TP was disambiguated and considered to be 3.17 min in the semi-targeted analysis of sample TIM2982 (**Figure 3.14**). The RT error in Filter II was ± 0.45 min, which validated the correlation between the results of the forensic sample analysis and those of the pure standard injection.

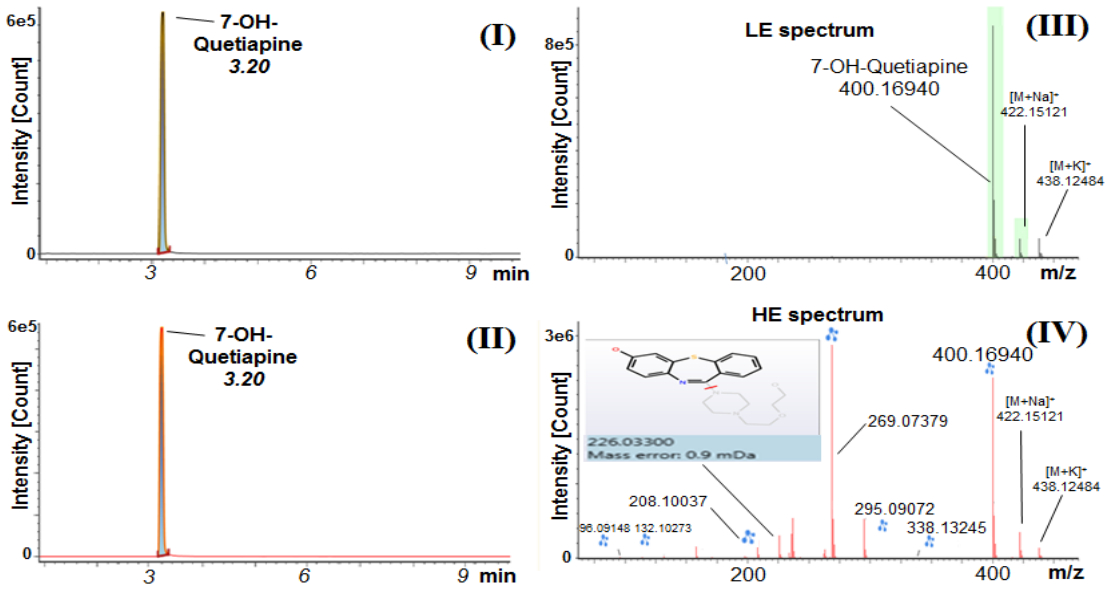

Figure 3.15 *UNIFI1.7™ "Analysis Method" window of detection for 7-OH-quetiapine in a whole blood at 20 ng/mg.* **(I)** *TIC evidencing the spiked reference standard with a high intensity peak;* **(II)** *XIC for the mass 400.16940 m/z, correspondent to the protonated 7-OH-quetiapine;* **(III)** *Low collision energy spectrum for the elution time of 3.20 min;* **(IV)** *High collision energy spectrum for the chromatographic peak at 3.20 min, displaying 7 product ions. The molecular structure of fragment with mass of 226.03300 m/z is highlighted and illustrated by the software.*

Figure 3.15 (**III**) reveals the protonated molecule, the Na^+ and the K^+ of 7-OH-quetiapine, which further enhanced the credibility of 7-OH-quetiapine identification.

Figure 3.15 (**IV**) presents the high collision energy spectrum revealing 7 product ion. Among them there are 269.07379 m/z and 208.10001 m/z, which were also present in **Figure 3.14** (**IV**), within the permited mass error of 3 mDa. Additionally, the fragment with the mass of 226.03300 m/z, with the attached molecular structure, was also present in sample TIM2982, with an AM of 226.03256 m/z.

With this example it was proved that the methods´ semi-targeted screening is a viable option for identification of compounds not present in commercial libraries or to which pure standards are of complicated acquisition, such as metabolites.

Notwithstanding, the pure standard and the information that RT provides are indispensable for the unambiguous identification of the compound semi-targeted identified.

Another group of small molecules to which reference standards are of difficult access are designer drugs. Next section provides the example of the designer drug that was semi-targeted identified by the screening method herby developed.

3.2.7 Designer drug identification

In **Figure 3.16**, it is presented the "Analysis Method" of O-acetylpsilocin (4-AcO-DMT) which was semi-targeted identified in sample TIM3034.

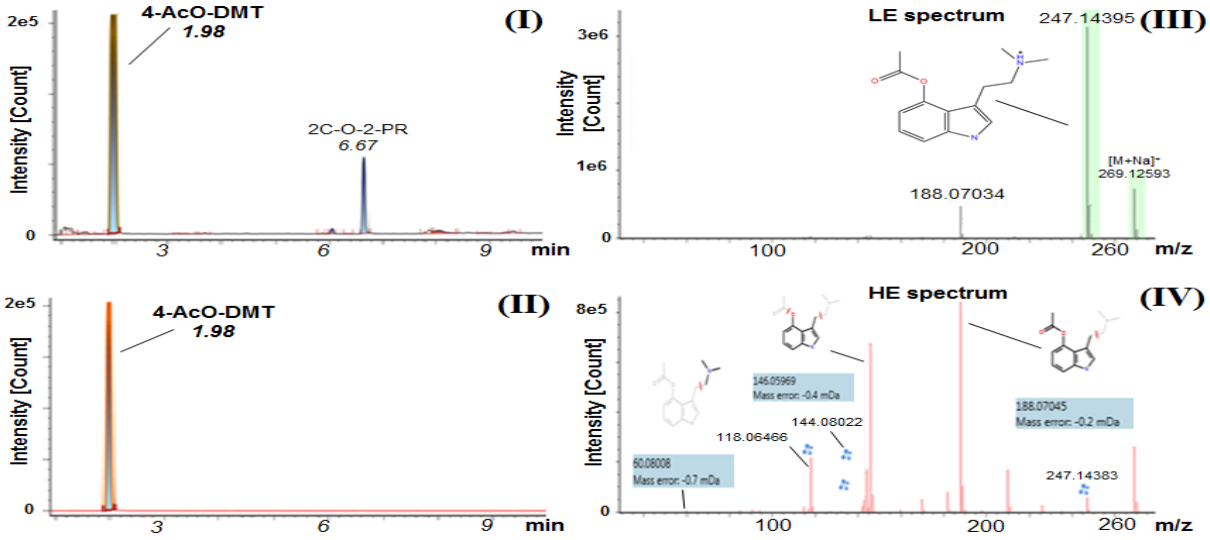

Figure 3.16 *UNIFI1.7™ "Analysis Method" window of detection for 4-AcO-DMT in sample TIM3034.* **(I)** *TIC evidencing the suspect under analysis with a high intensity peak, and 2C-O-2-PR (6.67 min);* **(II)** *XIC for the mass 247.14395 m/z, correspondent to the protonated 4-AcO-DMT;* **(III)** *Low collision energy spectrum for the elution time of 1.98 min;* **(IV)** *High collision energy spectrum for the chromatographic peak at 1.98 min, displaying 6 from the 11 product ions identified. The molecular structure of the fragments with mass o of: 60.08008 m/z, 145.05969 m/z and 188.07045 m/z are highlighted by the software.*

As in the case of 5-MeO-DIPT (see **Figure 1.12**) 4-AcO-DMT is a tryptamine derivate, synthetically produced, designer drugs. It was listed in the *UNDOC* database of "new psychoactive substances". It is the acetylated form of psilocin (structure also present in **Figure 1.12**), which is a natural occurring alkaloid present in the *psilocybe semilanceata*, colloquially known as "magic mushroom" [126].

Figure 3.16 (**I**) provides the TIC of sample TIM3034, displaying the peak relative to 4-AcO-DMT with an intensity of 200000 counts and eluting at the minute 1.98. The other semi-targeted identified compound (2C-O-2PR) with a RT of 6.67 min was classified as FP.

Figure 3.16 (**II**) illustrates the XIC for the mass of 247.14395 m/z, characteristic of O-acetylpsilocin. It didn´t display the common RT ambiguity that characterizes the semi-targeted screening, as an individual peak was exclusively illustrated. This enhanced the credibility of the hit.

Figure 3.16 (**III**) displays the low collision energy spectrum, evidencing the designer drug´s molecular structure attached to the spectrographic peak relative to the protonated molecule. Additionally it is displayed the Na^+ adduct, which meet the **identification criteria** listed in **section 3.1.1**. Also, it presents the fragment with mass of 188.07034 m/z. Given that the analytes´ molecule is prone to fragmentation, it is acceptable to admit that the molecule could have produced product ions at a collision energy of 4 eV.

The high collision energy spectrum **Figure 3.16 (IV)** provides the most substantial evidence to classify the hit as a UP due to the amount of fragments produced (see **Figure 3.17**). In the **Figure 3.16 (IV)**, there are present 6 of the 11 product ions proposed by *UNIFI1.7™*. To three of them, 60.08008 m/z, 146.05969 m/z and 188.0745 m/z, the respective ionic structures are illustrated, evidencing cleavage in the most electrically unstable bonds of the parent compound´s molecular structure.

Component Summary ▾

	Component name	Observed RT (min)	Identified High Energy Fragments
1	Dinortramadol	8.06	0
2	2C-O-2 PR	6.66	3
3	4-HO-DET	6.05	2
4	4-AcO-DMT	1.98	12

***Figure 3.17** "Component Summary" of the 4-AcO-DMT and 3 other suspect hits.*

In sum, all chromatographic and spectral information provided information to classify the hit as a UP. However, for non-doubtful classification, the respective pure standard need to be run to gather the RT and compare it with that obtained in this case: 1.98 min. Unfortunately, the reference standard wasn't available at the time of the analytical procedure.

Notwithstanding, the objective of semi-targeted identification of a designer drug was accomplished.

3.3 Non-targeted screening

For this screening method, accounting the results obtained from targeted and semi-targeted screenings, it was made a selection of samples in which the hypothetical presence of a designer drug was more probable.

12 cases were selected for further non-targeted toxicological screening: TIM2938, TIM2946, TIM2952, TIM2955, TIM2978, TIM2998, TIM3012, TIM3017, TIM3038, TIM3044, TIM3045 and TIM3068.

The procedure was divided in 3 stages:

- Search for unique peaks;
- Search for molecular formula;
- Molecular structure elucidation.

Along with the "identified" filter, both software filters developed in **section 3.1.5** contained parameters too wide to provide an analyzable amount of candidate peaks. Hence, a new Filter III was employed and its parameters are present in **Figure 3.18**.

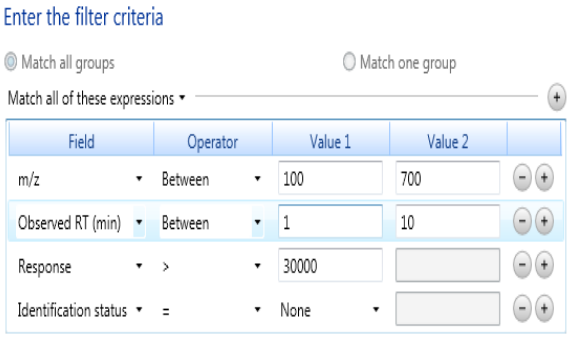

Figure 3.18 *Software Filter III set parameters*

By convention, small molecules are those that correspond to a molecular mass of less than 1000 Da. However, the considered mass interval, for non-targeted screening, was between 100 and 700 m/z. This was justified because of the large amount of unknown peaks detected in a single injection and by the fact that, in congruence with de Castro *et al.* [120], all the peaks eluted outside that mass interval corresponded to interferences derived from ME.

The retention time was from 1 to 10 minutes because after 10 minutes it was common the elution of a considerable amount of non-PTRC interferences. By trial and error, it was concluded that a threshold in the response should be implemented. Hence, exclusively hits with more than 30000 detector counts were considered. The "Identification status" field was set to "None" as no reference software library was used at this stage of the overall screening method.

Additionally, specific software features such as "mass difference elucidation tool" and "elemental composition elucidation tool", used for candidate peak interpretation, will be presented.

3.3.1 Search for unique peaks

The application of Filter III provided an exponential reduction of the putative candidate list, from several hundreds to exactly 40 hits, shared by the highlighted 12 cases. The results are provided in **Table 3.13** where a color filter was applied to correlate candidates with the same exact mass (limited to a mass error of 3 mDa) and retention time (RT error up to 0.02 min).

Table 3.13 *List of 40 software filtered candidates, with colorimetric results of peak uniqueness assessment based on accurate mass and RT.*

Case	Analyte	Response	RT (min)	Classification
TIM2938	Candidate Mass 229.1545	41077	1.04	Non-Unique
TIM2938	Candidate Mass 240.1595	58722	6.37	Non-Unique
TIM2938	Candidate Mass 254.1391	192159	6.37	Non-Unique
TIM2938	Candidate Mass 256.1549	41637	3.94	Non-Unique
TIM2938	Candidate Mass 259.0943	80156	6.37	Non-Unique
TIM2938	Candidate Mass 261.1103	30060	3.94	Non-Unique
TIM2938	Candidate Mass 354.0721	190537	5.70	UNIQUE 1
TIM2938	Candidate Mass 380.2075	63020	4.57	UNIQUE 1
TIM2938	Candidate Mass 517.1814	138521	6.37	Non-Unique
TIM2938	Candidate Mass 533.1539	31979	6.37	Non-Unique
TIM2946	Candidate Mass 248.1498	48781	1.03	Non-Unique
TIM2946	Candidate Mass 229.1555	79077	1.04	Non-Unique
TIM2952	Candidate Mass 310.3509	32925	8.59	Non-Unique
TIM2952	Candidate Mass 265.1590	181873	8.59	Non-Unique
TIM2952	Candidate Mass 229.1550	45104	1.04	Non-Unique
TIM2955	Candidate Mass 241.1550	31282	1.04	Non-Unique
TIM2955	Candidate Mass 229.1551	31783	1.04	Non-Unique
TIM2998	Candidate Mass 684.2047	321442	1.02	Non-Unique
TIM2998	Candidate Mass 246.1701	41248	2.31	Non-Unique
TIM3012	Candidate Mass 247.1082	67300	3.93	Non-Unique

Case	Analyte	Response	RT (min)	Classification
TIM3012	Candidate Mass 269.0901	33635	3.92	Non-Unique
TIM3012	Candidate Mass 364.0599	30969	3.05	Non-Unique
TIM3012	Candidate Mass 442.1032	125041	3.05	Non-Unique
TIM3017	Candidate Mass 265.1588	96736	8.60	Non-Unique
TIM3038	Candidate Mass 241.1541	33226	1.04	Non-Unique
TIM3038	Candidate Mass 246.1702	30238	2.61	Non-Unique
TIM3038	Candidate Mass 229.1550	52041	1.04	Non-Unique
TIM3038	Candidate Mass 232.1546	51069	1.43	UNIQUE 1
TIM3038	Candidate Mass 332.1405	32517	3.61	UNIQUE 1
TIM3038	Candidate Mass 332.2795	36965	2.70	UNIQUE 1
TIM3038	Candidate Mass 346.1198	31519	4.73	UNIQUE 1
TIM3044	Candidate Mass 241.1544	37491	1.04	Non-Unique
TIM3044	Candidate Mass 325.0713	30152	5.91	UNIQUE 1
TIM3045	Candidate Mass 248.1494	41754	1.03	Non-Unique
TIM3068	Candidate Mass 310.3513	32631	8.61	Non-Unique
TIM3068	Candidate Mass 265.1597	203323	8.61	Non-Unique
TIM3068	Candidate Mass 248.1500	42819	1.03	Non-Unique
TIM3068	Candidate Mass 229.1554	31950	1.04	Non-Unique
TIM2978	Candidate Mass 684.2033	64315	1.02	Non-Unique
TIM2978	Candidate Mass 398.1533	68763	6.57	UNIQUE 1

As results, 8 compounds were regarded with the classification of "UNIQUE 1". This signified that they passed the criteria of AM and RT uniqueness (results displayed in columns "Analyte" and "RT (min)", respectively). At this stage, from the initial 12 samples, only 5 (TIM2938, TIM2978, TIM2998, TIM3038 and TIM3044) were selected for further interpretation.

Extended investigation involved the attribution of fragment exact mass for each UNIQUE 1 candidate. The fragments were acquired from the HE spectrum of each unknown and only the 4 (if provided) with the highest detector count were considered in this analysis.

Figure 3.19 is an example of the procedure of allocation of product ions to the correct parent ion. It provides the spectral information of the candidate 325.0713 m/z from injection TIM3044.

Chromatograms (TIC and XIC) were not provided by the software, in non-targeted screening, due to the amount of hits that are detected. It´s presentation would require a great amount of computer power which would turn the analysis time ineffective. However, despite no graphical display of chromatographic results, *UNIFI1.7™* attributes the empirically acquired RT for each candidate. For the case of candidate in **Figure 3.19** the observed RT corresponded to 5.91 min (see **Table 3.13**).

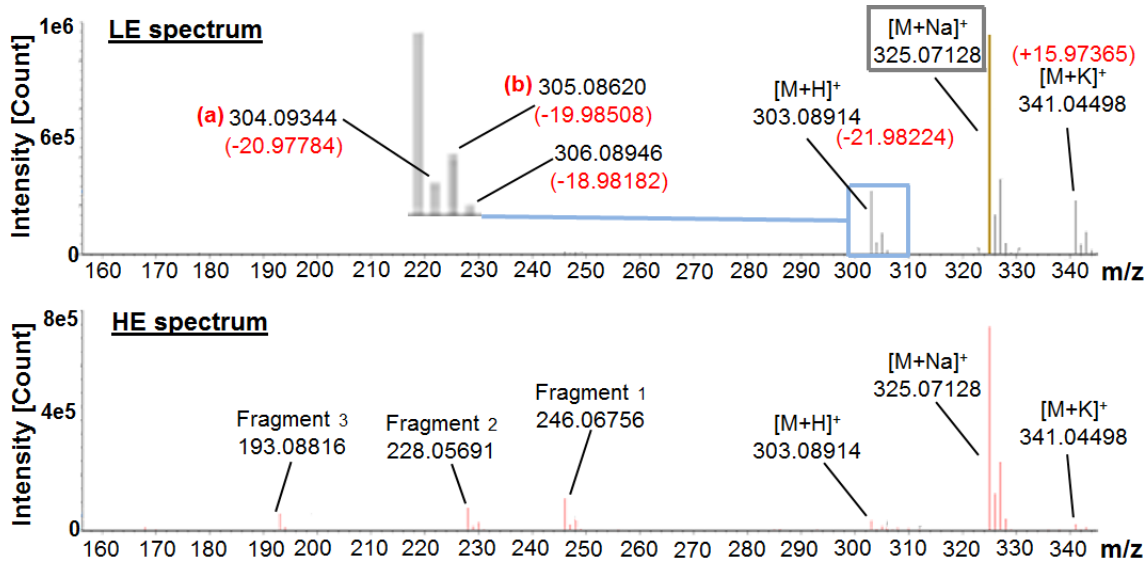

Figure 3.19 *Adduct identification and fragmentation allocation for candidate with mass of 325.07128 m/z, and. protonated molecule isomer identification.*

In the low collision energy spectrum it is possible to observe that the candidate in analysis, inside the grey box, was relative to the parent ion (M) associated with the Na^+ adduct. For this assumption it was of great contribution the software´s "mass difference elucidation tool", which information are displayed in red in **Figure 3.19**.

The proton, sodium ion and potassium ion have exact masses of 1.007276 m/z, 22.989219 m/z and 38.963158 m/z, respectively [127]. From that, the software was able to

calculate de differences between all the peaks (including the isotopes for each specie, exemplified by (a), (b) and the isotope with mass of 306.08946 m/z), which assisted in the identification of the spectral peaks. This was of extreme importance for the molecular formula and structure elucidations procedures.

Therefore, fragments 1, 2 and 3 were unambiguously regarded as product ions, as they increased considerably in quantity (Intensity [count]) from the LE to the HE spectrum. However, the peak at 303.08914 m/z, which could be confused with a fourth fragment of the candidate with 325.0713 m/z suffered a reduction in Intensity [count]. Hence it could not be appointed as a product ion, but instead as the protonated parent ion, as the "mass difference elucidation tool" revealed.

Furthermore, it was possible to identify the peaks 303.08914m/z and 341.04498m/z as the protonated molecule and the potassium adduct respectively, and the candidate 325.0713m/z corresponded to the sodium adduct.

This directs to the conclusion that all three peaks highlighted in the previous paragraph corresponded to the parent ion and the respective adducts of an unknown compound having an exact molecular mass of 302.081864 Da. However, this example describes a rare case where the highest MS peak corresponded to one of the adducts.

Additionally it is presented the isotopes for the protonated molecule. The isotopes (a) and (b) were of high importance in the molecular formula prediction effectuated in **section 3.3.2.**

This example also demonstrates the degree of complexity when performing reverse search through a non-targeted screening method.

The procedure was reproduced for the remaining UNIQUE 1 candidates and the results are presented in **Table 3.14**.

Table 3.14 *List of 8 UNIQUE 1 candidates with colorimetric results of candidate uniqueness assessment based on exact mass of fragments.*

Case	Analyte	RT (min)	Adduct	Fragment1	Fragment2	Fragment3	Fragment4	Classification
TIM2938	Candidate Mass 354.0721	5.70	H Na	138.05506	170.02705	126.05509	110.06011	UNIQUE 2
TIM2938	Candidate Mass 380.2075	4.57	H Na	275.12826	85.02851	191.10695	145.10137	UNIQUE 2
TIM3038	Candidate Mass 232.1546	1.43	H Na K	173.08074	144.10179	85.02811		UNIQUE 2
TIM3038	Candidate Mass 332.1405	3.61	H Na	288.15102	245.10866	314.13008	268.14444	Non-Unique 2
TIM3038	Candidate Mass 332.2795	2.70	H	288.15102	245.10866	314.13008	268.14444	Non-Unique 2
TIM3038	Candidate Mass 346.1198	4.73	H Na K	217.04049	245.07194	287.06942	298.20063	Non-Unique 2
TIM3044	Candidate Mass 325.0713	5.91	Na H K	246.06756	228.05691	193.08816		UNIQUE 2
TIM2978	Candidate Mass 398.1533	6.57	H Na	253.07947	221.10752	279.09452		UNIQUE 2

At this stage, fragmentation pattern was introduced as an additional criterion that was applied to narrow down the list of candidates. As it is possible to attain in the color tags of

Table 3.14, three compounds, with the exact masses of 332.1405 m/z, 332.2795 m/z and 346.1198 m/z shared at least one fragment. As for that they were excluded from further molecular formula elucidation (discussed in **section 3.3.3**).

Despite the fact of two candidates, from sample TIM2938 with masses of 380.2075 m/z and 232.1546 m/z, shared a common fragment, they were considered for further analysis. This resided in the fact that the common fragment, 85.02851 m/z (red), was accounted as the most commonly detected mass in the HE spectra (see **Figure 3.2**). Hence, an attempt to identify its ionic structure was considered of interest.

3.3.1.1 Co-eluting peaks

In **section 3.3.1** it was referred that identical RT between two candidates was enough to exclude them from further analysis. This criterion was adopted, because co-eluting peaks, within 0.02 minutes, provided complicated HE spectra, as illustrated in **Figure 3.20**. There, it is exemplified the candidates with exact masses of 517.1814 m/z and 533.1539 m/z, from the case TIM2938 and eluting at min 6.37.

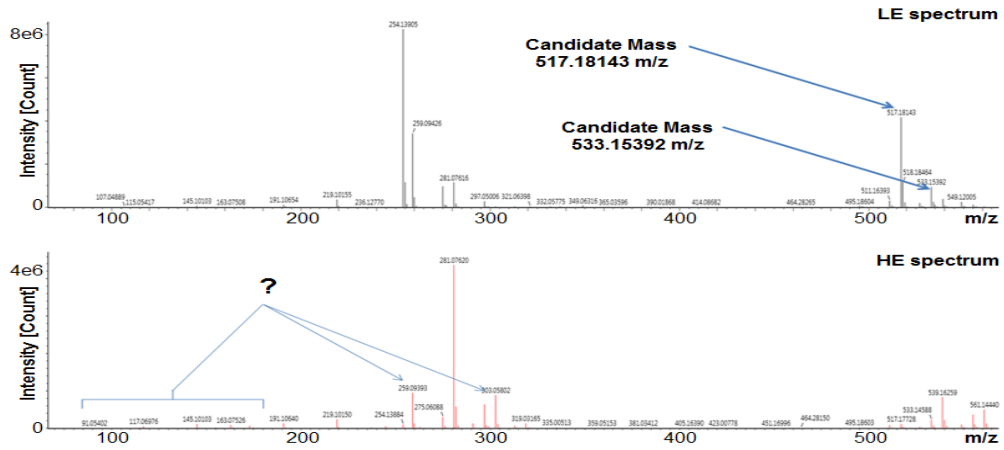

Figure 3.20 *HE and LE spectra of co-eluting candidates: 517.18143 m/z and 533.1592 m/z.*

In this case, the co-eluting peaks are indicated in the same LE spectrum, which is abnormal and was not verified in any targeted and semi-targeted hit classified as UP. Hence, in the high collision energy spectrum of **Figure 3.20**, the peaks signaled with an interrogation mark weren´t possible to be individually correlated to either the candidate with mass of 517.18143 m/z or to the candidate with mass of 533.1592 m/z. So, they were excluded from analysis. From the 40 initial candidates, 13 candidates were discarded from analysis solely in the basis of spectra that resulted from co-elution.

Each DIA complete cycle time was of 0.424 seconds, which corresponded to 0.007 min. This signified that, at every moment of the considered detection interval (from minute 0.9 until minute 13) data was independently acquire, not covering small intervals of 0.024 s, between each DIA scan.

Hence, the problematic did not reside in the software nor in the computational power, but in the resolving power of the instrument. As chromatographs with more resolution are needed in order to provide a more effective peak separation, so these co-eluting peaks may be considered for molecular formula allocation.

However, the development of micro-channeling technology that serves as background of LC instruments as reached a plateau. This stagnation may be explained by the uncontrollable nano-effects that characterize columns with particles under 1.7µm [128].

As a mean to circumvent this problematic, the adoption and development of a new dimension, in terms of analyte separation, should be employed.

Ion mobility spectrometry (IMS) combined with MS, a relatively new separation technology with applicability in trace chemical detection and analysis, fits the criteria to be a feasible option to overcome the resolution limitations of LC [129].

In its simplest scenario, IMS consists in the acceleration of an ion by an electrostatic field in one direction. Collisions with background gas molecules or other species hinder the ion´s path in the electric field. So, an ion is accelerated by the electric field and collides with neutral molecules (devoided of fragmentation) losing all of its momentum, and is subsequently accelerated again. Hence, the preponderant criteria in this analyte separation is the ionic structure´s size and conformation [129].

Despite promising, this technology is still futuristic and further development and validation are necessary [129] to confirm its ability to provide a separation platform, with enough resolution to match the computation power of the detector employed in this thesis.

3.3.2 Search for molecular formula

At this stage, the molecular formulas of the 5 candidates classified as UNIQUE 2 were probabilistically obtained using the software's "elemental composition elucidation tool". The candidate with mass to charge ratio of 325.07128 m/z continues to serve as example of the procedure. Hence, **Figure 3.21** illustrates the operating settings of the tool, with comprehensive legends.

After identification of the fragments that characterized the candidate, the intended mass of the protonated ion was plotted in the "m/z" field (**Figure 3.21 (I)**). It is relevant to state that *UNIFI1.7™* considers the mass of the sodium adduct when predicting the molecular formula of the candidate. For this purpose it was used the exact mass of 303.0891 m/z instead of 325.07128 m/z, since the candidate have been detected as a sodium adduct in the first place. This is of importance as the molecular structure elucidation procedure

(**section 3.3.3**) was based on data correlation with reference libraries present in websites. These libraries do not store molecular formulas/AM of adducts but exclusively of the protonated molecule.

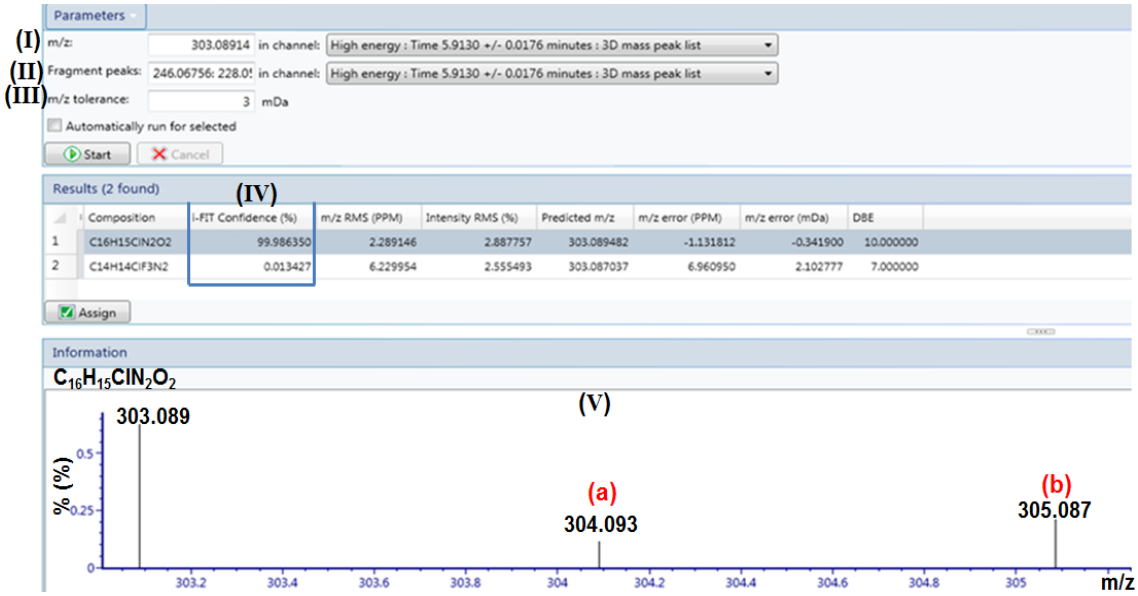

Figure 3.21 *Description of software´s "Elemental composition elucidation tool" for molecular formula prediction o the protonated molecule of sample TIM3044´s candidate.*

The AMs of the 3 identified fragments (see **Figure 3.19**) were introduced in the "Fragment peaks" field **(II)**. The mass deviation accounted for in the elemental composition elucidation had the maximum mass deviation of 3 mDa, as observed in "m/z tolerance" field **(III)**.

With the three parameters selected, the calculation of a probabilistic molecular formula was initiated.

The most relevant criterion of this non-targeted analysis stage was the "i-FIT Confidence (%)", relative to column **(IV),** which algorithmically provided molecular formulas ($C_{16}H_{15}ClN_2O_2$ and $C_{14}H_{14}ClF_3N_2$, in this case) by correlating the predicted isotope pattern, present in graph **(V)**, with the experimentally measured in the LE spectrum of **Figure 3.19**. The "i-FIT" calculates the degree of similarity of the isotopes (a) and (b) acquired in both sources. In the case of the protonated ion of the candidate 325.07128 m/z (303.0891 m/z) the most probable molecular formula was $C_{16}H_{15}ClN_2O_2$ with an "i-FIT" of 99.9 %, and from the two displayed in the results (**Figure 3.21** (**IV**), it was the one used for further structure elucidation of the intended candidate.

This procedure was repeated for the 4 remaining UNIQUE 2 candidates and the results are presented in **Table 3.15**.

Table 3.15 *Proposed molecular formulas for UNIQUE 2 candidates.*

Case	Analyte	Adduct	i-FIT Confidence (%)	Molecular Formula	Compound
TIM2938	Candidate Mass 354.0721	H+; Na+	38.7	C11H22N3O2P3S	Irrelevant
TIM2938	Candidate Mass 380.2075	H+; Na+	69.9	C17H30FNO7	I
TIM3038	Candidate Mass 232.1546	H+; Na+; K+	99.9	C11H21NO4	II
TIM3044	Candidate Mass 325.0713	Na+; H+; K+	99.9	C16H15ClN2O2	III
TIM2978	Candidate Mass 398.1533	H+ Na	37.8	C20H24N5PS	Irrelevant

Only the compounds with an "i-FIT" Confidence above 50% were submitted to structure elucidation. As for that only the compounds **I**, **II** and **III** were considered for further structure elucidation.

3.3.3 Molecular structure elucidation

At this stage, two databases available online were employed for structure elucidation: *mzCloud*™ and *ChemSpider*™. The former was the first choice for molecular structure identification and the latter was used if not sufficient data was achieved in *mzCloud*™.

Additionally, if for a molecular formula, more than one chemical structure were displayed in the *ChemSpider*™, *ChemBioDraw Ultra 12.0*™ was utilized for fragmentation prediction. Hence, the latter contributed for the identification of the correct hit from the options provided in *ChemSpider*™. Both *mzCloud*™ and *ChemBioDraw Ultra 12.0*™ approaches had as reference the measured product ions, presented in **Table 3.14**, page 87.

In this section it is presented the final results from structure elucidation of compounds I, II and III, listed in **Table 3.15**.

Compound I

The candidate from sample TIM2938 with mass of 380.2075 m/z was not listed in neither the considered on-line databases and for such it was excluded from the analysis. This result suggests that candidates with an "i-FIT" value under 70 %, should not be considered for further analysis.

Compound II

The structure of candidate from sample TIM3038, with mass of 232.1546 m/z, was elucidated *via mzCloud*™*.* The website search criteria were those listed in **section 2.5.3**, with 232.1545 m/z plotted in the "Precursor m/z" field and the masses 85.0211 m/z, 144.10179 m/z and 173.08074 m/z plotted in the "Peak list" field.

The results are illustrated in **Figure 3.22**, were it is possible to observe that, respecting the employed margin of 0.009 Da, the structures for the precursor and product ions were elucidated.

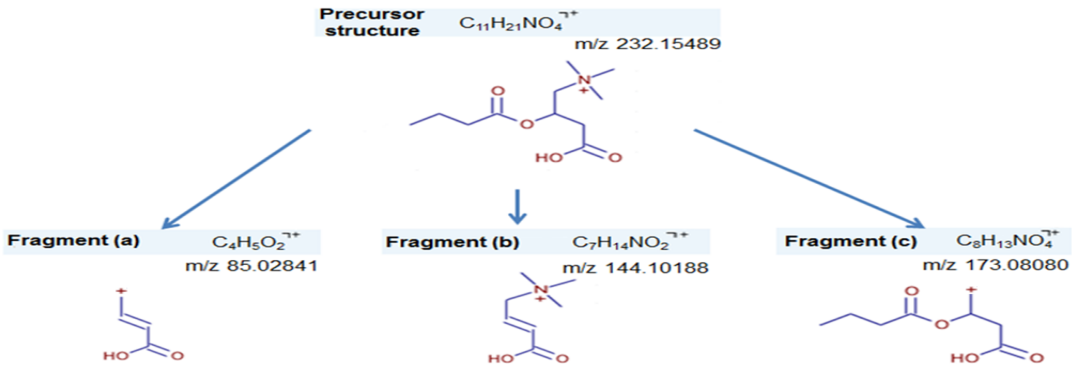

Figure 3.22 *mzCloud™ structure elucidation of candidate with mass 232.1546 m/z.*

With the reverse search completed (from the candidate´s exact mass to the acquirement of the molecular structure) the molecular formula was introduced in the search engine of *ChemSpider™*, displaying 369 isomers for $C_{11}H_{21}NO_4$. However, with the *mzCloud™* results, it was straight forward to find the *ChemSpider™* file containing the correct compound and relevant information. In this case it corresponded to that with ID number of 388877.

Compound II was identified as O-butyryl-(L)-carnitine, which is an acyl ester of carnitine or acylcarnitine [130].

Carnitine and its acyl esters are essential compounds for the metabolism of fatty acids. These compounds are present in tissue at relatively high concentration, typically between 0.2 and 6 mmol/kg, with most being present in the heart and skeletal muscle [130]. Acylcarnitines are a large group of endogenous compounds and, in addition to compound II there are, for example, *O*-hexanoyl-(L)-carnitine, *O*-octanoyl-(L)-carnitine and O-decanoy-(L)-carnitine [130]. As the name suggests, they vary only in the number of carbons present in the aliphatic chain. Hence, fragment (a) and fragment (b) in **Figure 3.22**, are unchangeable in all varieties of acylcarnitines. This justified the fact that fragment (a) was the most commonly detected fragment in the herby developed screening method (see **Figure 3.2**, and **Table 3.14**).

Despite belonging to a non-PTRC, this hit proved the concept of non-targeted screening. A peak was identified from a chromatogram without any library support or previous knowledge about what was being analyzed.

Additionally, and most importantly, as fragments (a) and (b) from **Figure 3.22** are non-variable in this abundant group of endogenous compounds, they can be used to reduce the number of non-targeted hits, by excluding from analysis all the unknown peaks that include the exact masses of 85.0211 m/z and 144.10179 m/z in their HE spectra. The process is similar to that adopted in the targeted screening in order to exclude compounds present in the “excluded” library (see **section 3.1.4**). This procedure may be applied to other

endogenous compounds in whole blood, so the sensitivity of the method may increase, because with less non-targeted hits to account for, a lower response threshold (lower than 30000) can be plotted in Filte III.

Compound III

$C_{16}H_{15}ClN_2O_2$ was the molecular formula elucidated by *UNIFI1.7™* from the candidate´s protonated molecule AM of 303.0891 m/z. When introduced in the search engine of *mzCloud™,* the formula failed to provide any hit. However, it produced 1232 hits in the *ChemSpider™* database.

In order to elucidate the molecular structure it was used *ChemBioDraw Ultra 12.0™* for the simulative study of the candidate´s fragmentation pattern, in the light of the fragmentation rules referred by Niessen [131]. The molecular ion prediction was based upon the structures proposed in the referred website database and in comprehensive targeted screening results for the *antmortem* traffic case TIM3044.

Ionic structures were proposed, which satisfied candidate III and the respective fragments´ measured exact masses. The results are illustrated in **Figure 3.23.**

The three fragments provided by the *UNIFI1.7™´s* HE spectrum had 246.06756 m/z, 228.05691 m/z and 193.08816 m/z. as exact masses. The illustration depicts the exact masses provided accordingly to *ChemBioDraw Ultra 12.0™*. Hence, for rigorousness, the permitted deviation from this platform relatively to *UNIFI1.7™* measurements was 9 mDa.

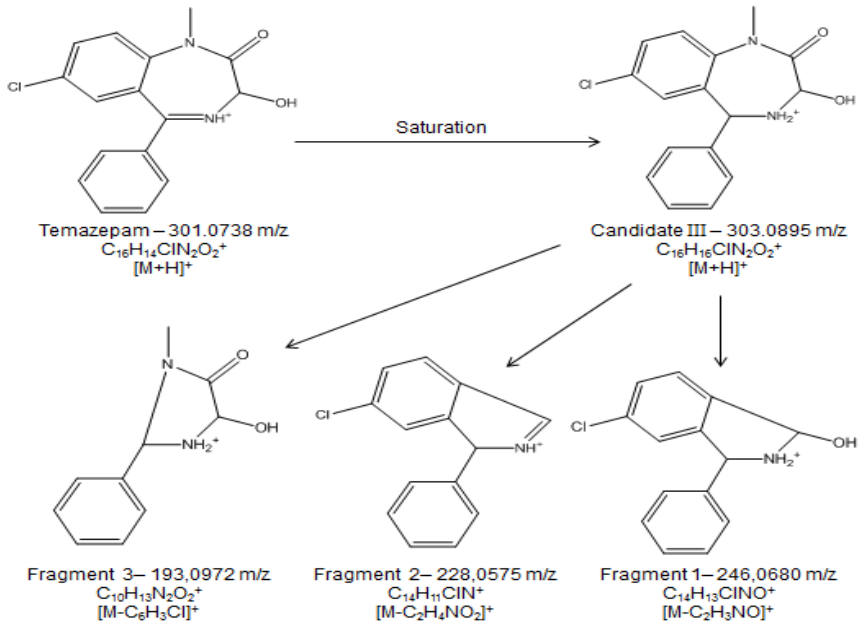

Figure 3.23 *Metabolic pathway of temazepam and fragmentation pattern of candidate* III.

Through a lengthy attempt and error procedure, it was concluded that compound III was probably related to the targeted screening hit correspondent to temazepam, a psychoactive benzodiazepine prescribed drug [132].

The metabolic pathway of temazepam is known to be incomplete and only two metabolites had been identified so far. One corresponds to a major metabolite resulting from the conjugation of glucuronate or sulfate with the oxygen from the ketone. The second results from the demethylation of nitrogen of the precursor or from the nitrogen of already formed O-conjugated TPs [132].

Compound III probably corresponds to an additional non-toxic temazepam metabolite (not identified in literature) originated from the saturation of its parent compound. Its non-targeted identification enhance the credibility of the CP hit attained by both the developed screening method and parallel confirmatory MS/MS methodologies, in which results are listed in the LIMS database. According to the latter, the precursor molecule (temazepam) was quantified at 0.2 mg/Kg, a concentration 10 times higher than its LTC. Hence, high degree of metabolization was expected.

The previously highlighted assumption is supported by the fragments´ structures elucidation, which was guided by the rules of fragmentation of even-electron ions [131].

According to the nitrogen rule, an odd-electron ion with and odd number of nitrogen atoms should have an odd m/z, whereas an even-electron ion with an odd number of nitrogen atoms should have an even m/z. By exclusion of parts, as the elucidated candidate´s structure has an odd m/z (303.0895) and an even number of nitrogen atoms, it can be stated that it corresponded to an even electron ion. This meant that it satisfied the criteria of a protonated ion generated by an ESI, enhancing the credibility of the compound III´s hit [131].

Parity rule or even-electron rule states that upon fragmentation the electron pairs remain intact [131]. So the counterparts of fragments 1, 2 and 3 of **Figure 3.23**, corresponded to a neutral loss molecule. This was fundamental for the understanding of the saturated bounds resulted from the conjugation of the unpaired electrons at each cleavage site. This was observable in all three fragments.

As a consequence, the loss of a neutral molecule from an even-electron ion (that conserves the intended product ion as even in terms of m/z) is far more likely than the loss of a radical, which would originate and odd-electron fragment ion. Both fragments 1 and 2 respected this assumption. However, fragment 3 had an odd m/z. This was due to the fact that the most important deviations of the even-electron rule involves the loss of relatively stable radicals from aromatic ring systems, such as $NO_2^{\bullet}$, $NO^{\bullet}$, $Br^{\bullet}$ and the one of interest, $Cl^{\bullet}$ [131]. Therefore, fragment 3 continued to be congruent with what was expected from the rules stipulated in the fragmentation of even-electron ions.

Another important criterion that validated the proposed fragments as a plausible hit was the fact that even-fragmentation in protonated molecules involves carbon-heteroatom cleavage [131], which was observable in all 3 fragments.

Additionally, all the fragments provided by the *ChemBioDraw Ultra 12.0™* where within the mass error of 9 mDa, stipulated previously.

With this it can be stated that compound III has a high probability of having the structure proposed in **Figure 3.23**. However such in the previous semi-targeted screening of this 3 staged screening methodology, the pure standard is a necessity for unambiguous identification of the analyte, which was not available due to non-acknowledgement of the non-targeted identified temazepam metabolite.

3.4 Comparison with literature

This section provides the comparison between the results achieved by the STA developed in this project, with those published by nine research teams covering the two dualities stressed in the thesis´ literature review (see **section 1.6**): STA based on product ion spectra *vs* GUS based in HR/AM acquisition; and DDA *vs* DIA.

The screening capabilities of the screening methodology hereby developed will be comparatively assessed.

When comparing the chromatograms and spectra of results in Lafaye, *et al*. [7] (see **Figure 1.31**) with the “Analysis Method” window from the software employed in this thesis (*e.g.* see **Figure 3.1, Section 3.1.3**), it is possible to highlight some distinctions that reveal the technological gap of 10 years between the two screening methodologies. Therefore, using the data acquisition as the comparison argument, the red double edged arrow in the **Figure 1.31**, page 36, evidences the absence of an algorithm to correctly align spectrums **(II)** and **(III)**. Additionally, it is possible to attain that the peak correspondent to ≈385 m/z has a distinct nominal mass between **(II)** and **(III)**, not to mention the difference of exact masses.

In the same context, the DIA screening based on HR/AM acquisition, developed in this project, employed algorithms that correctly align spectra peaks of parent, metabolite and fragment ions generated from 2 distinct collision induced dissociation (CID) energies (see **Figure 1.30**, page 34). Additionally to this virtual spectra alignment, which ultimately gave the upper hand for unambiguous analyte/candidate identification, *UNIFI1.7™* was able to correlate the accurate mass (up to 4 decimal houses) of spectral peaks between both acquisition functions. This way, the resolution was much superior to that of nominal mass based screening methods, such as the one employed by Lafaye, *et al*. [7].

Dularent, *et al*. [4] STA based on product ion spectra provided a successful application of a screening involving an artificially intelligent data acquisition (DDA in this case). However, the methodology was prone to a certain degree of impairments in terms of sensitivity, as the standard LOD was of about 10 ng/mL. Whereas, in this project´s developed screening method, it was proved that the LOD spammed from 1 ng/mL to 100 ng/mL for 198 representatives PTRCs (see **section 3.1.2** and **Appendix I**). Additionally, in order to increase the sensitivity for some compounds, the author ran intended samples, in a single polarity, exclusively in MS^2. However, the resulting specificity was so conditioned that the confirmatory data wasn't possible to be recorded in the MS^3 scan. So, for cases where very high sensitivity was needed, a parallel method was required [4].

In contrast, despite the targeted screening results of this thesis had been compared with those of other instruments, ran in parallel at RKA, the aim of this project was to develop an all-purpose screening method. Therefore, analyses made in a way that solely contemplated a particular analyte, due to sensitivity threshold, were categorically discarded. However the all-purpose screening capability was impaired due to sensitivity deficiency regarding the detection/identification of morphine and compounds exclusively detectable when negatively ionized.

With an example of an STA based on product ion spectra ,but without resource to any artificially intelligent data acquisition, Liu, *et al.* [98] were able to implement a novel fragmentation approach which consisted in providing voltage ramping and broadened mass window for activation (or fragmentation width) [98].

However, this paper failed to provide a representative amount of real *postmortem* cases, as only 12 were reported. Additionally, despite being considered toxicologically representative by the author, in all of the cases opiates were identified, which may indicate that the sample selection wasn't representative for the whole spectrum of cases generally screened in *postmortem* toxicology [98].

For comparative purposes, it is relevant to restate that the method relative to this thesis covered 55 real *antemortem* and *postmortem* cases in which the whole blood samples were the representative biological matrix. Additionally, the DIA mode providing the parent (precursor) ion spectra at 4 eV and the ramping voltage at the CID cell was also a feature of the software/instrument used in this thesis. However this was exclusively applied in the high energy fragmentation function, which maximum voltage reached 40 eV.

Polettini´s research group employed a GUS based on HR/AM detection, which allowed them to explore the reproducibility of compound accurate molecular mass to the extent of assembling a targeted database containing 50500 toxicological relevant compounds and their metabolites. The massive database was based on *ChemSpider™* which contains more than 30 million compounds [3, 106].

On its image, in the present thesis it was defined a strategy similar to that of Polettini and associates, as the RT was also not considered for the semi-targeted screening, due to the fact that it is too irreproducible between instruments and even between trials in the same instrument [3, 106]. Molecular formula was the criterion of choice for semi-targeted and non-targeted screenings in this thesis context.

Comparatively, the main advantage of this work resided in the ability of *UNIFI1.7*™´s "i-FIT" tool to correlate AM of precursor and respective fragments with molecular formulas. This provided an additional dimension for the identification of unknowns, such as O-butyryl-(L)-carnitine and the saturated transformation product of temazepam (see **section 3.3.3**)

As in the case of this thesis, Lee *et al.* [2] reported a "real-time" HR/AM acquisition of low and high fragmentation spectra in the same analysis. The author considered exact mass determination to four decimal places, *per se*, the main criterion for achievement of adequate specificity. However this thesis´ filter development results suggested that AM is not enough and further criteria, such as mass error and RT error thresholds, had to be adopted in order to narrow down the FP frequency from a maximum of 89 % ("Identified" filter) to a minimum of 68 % (Filter I).

Pedersen, *et al*. [6] reports a methodology that functioned as the precursor of the STA of this thesis. The usage of automated SPE in sample preparation instead of automated PPT, the sample volume of 200 µL (in place of 100 µL of whole blood) needed for sample preparation and the software used for data treatment (*ChromaLynx XS*™ alternatively to *UNIFI1.7*™) were the main differences between both methods [6].

The author carried out STAs of 1335 authentic forensic traffic cases by screening for the intended 256 drugs. A number greater and more representative than the 55 (*antmortem* and *postmortem*) forensic cases covered in this thesis [6].

This thesis gave particular importance to LOI (extensively dependent on provision of fragmentation information) having Pedersen´s compound selection as a reference for the evaluation studies performed in this project [6].

Therefore, in terms of sensitivity studies, this thesis implied exclusively the LOI (almost fully reduced to LOF) determination for 198 compounds (an extract of Pedersen *et al.* database of 256) from those 1030 present in the commercial targeted library.

Despite being comparatively more accomplished in what regards the screening for targeted analytes, this literature example failed to explore the RP of the instrument to the extent of performing semi-targeted and non-targeted screenings, which are provided in this project (see **section 3.2** and **section 3.3**, respectively).

Humbert *et al.* [62] provided an example of a rare case in which a STA based on product ion spectra, associated with an artificial intelligent data acquisition (DIA in this case).

In comparison with this project, Humbert´s nominal mass DIA approach provided a broader study platform for further characterization of intended compounds. This was due to the fact that this literature was based on fragmentation pattern displayed in up to 10 times more spectra (with different CID energies) than those provided in this thesis [62].

Paul *et al.* [109] is a very recent publication concerning the employment of a STA based on HR/AM detection with application of a non-targeted DDA.

Similarly to the method developed in this project, Paul, *et al*. [109] procedure had the limitation of not providing quantitative nor semi-quantitative results. Hence it was missed an important criterion for the non-dubious identification of both targeted compounds and drug candidates [109].

On the other hand, this thesis' method encompassed a "true" non-targeted screening, in which no initial information on the candidates was available. Hence, instead of giving prominence to information provided by literature or operators' knowledge about metabolism/fragmentation, in the project herby presented the identification was mainly dependent on spectra deconvolution algorithms. These typically revealed chromatograms with several thousands of peaks in just an individual blood sample. Subsequently, AM, isotope patterns and fragment ions of candidates were accounted for by the "i-FIT" tool, provided by the software (*UNIFI1.7™*), in order to obtain molecular formulas from measured exact masses. From the correct molecular formula, tentatively identification of molecular structure followed.

The Finnish research group headed by Ojamperä was, in the last decade, the most committed in the contextualization of HR/AM acquisition methodologies into the ambit of STA.

The semi-targeted database assembled in this project (**section 3.2.1**) was assembled in the lighting of this author's work, although with some additional features, made available today through computational technology advances, and with the knowledge that AM and isotope pattern alone are not sufficient for unambiguous compound identification [111].

The screening technique developed in this thesis was extensively based on their findings, but with a more capable parent ion fragmentation approach.

Following up Ojampera's group findings, this study attempted to elucidate the importance of fragmentation pattern for targeted analytes identification and, most innovatively, for the elucidation of the molecular structure of PTRCs candidates in actual blood samples of forensic cases.

4 CONCLUSION AND FUTURE PERSPECTIVES

4.1 Conclusion

The method´s targeted screening provided an overall sensitivity lower than other LC-MS/MS methods employed in RKA, highlighting morphine as the most problematic PTRC to be screened for. On the other hand, it made available a wider analytical range, screening for 1030 basic small molecules in a single run, which revealed the method´s importance in the guidance of further quantitative STAs. 103 PTRCs, distributed throughout 231 detector hits, were identified. 50 compounds more than in all the methods ran in parallel in the RKA, being 112 identified hits exclusive to the developed targeted screening method.

The software Filter I provided an option to dramatically reduce the analysis time to a maximum of 20 min, for PTRC rich forensic samples, revealing the importance of the filtering parameters for a time efficient GUS.

The concept of semi-targeted was proved through the identification of 3 suspect PTRCs (active metabolites) to which convenient pure standards were available.

UNIFI1.7™ fragmentation prediction feature was evaluated by comparison with product ion prediction from *ACD/MS Fragmenter™*, and results were satisfactory.

Globally, 15 suspect compounds were identified by semi-targeted analyzing all 55 forensic samples. The compounds were distributed through 21 suspect hits. One hit with a particularly high probability of being a true positive corresponded to a designer drug, but the pure standard wasn't available for unambiguous identification.

A non-targeted screening methodology was developed revealing lack of sensitivity in the analyzed 12 samples. It also suggested that at this stage, non-targeted screening is too

laborious for a time effective STA. Two compounds were structurally elucidated by the non-targeted method, contributing for the methods proof of concept.

In what concerns the total amount of positively identified hits by the developed method, 90.9% corresponded to targeted screening, 8.3% to semi-targeted screening and 0.8% to non-targeted screening.

4.2 Future perspectives

In the 55 samples analyzed, a cause of death corresponded to phenobarbital poisoning. In order to obtain results for barbiturates, a run in negative mode ESI should be performed to verify the method´s sensitivity for this group of PTRCs.

As for semi-targeted screening, *UNIFI1.7*^{TM} capability of fragmentation pattern prediction was validated. Hence, in addition to the 61 imported molecular structures, the remaining 1331 should be drawn and implemented in the semi-targeted screening method.

Additionally, the reference standard of 4-AcO-DMT has to be run for provision of the analyte´s RT, so the designer drug hit in sample TIM3034 may be undoubtedly classified as a true positive. This would provide the ultimate proof of the methods´ feasibility in STA context.

The non-targeted screening is in its primordial stages of development. In order to increase the sensitivity of the method, the response threshold has to be dramatically reduced in the filtering parameters; or the sample volume (before preparation) has to be increased (*e.g.* from 1 µL to 5 µL). To avoid the latter resolution, a non-targeted “excluded” library should be assembled. This process would require the identification of the peaks (parent and product ions) respective to endogenous compounds of whole blood, such as the acylcarnitines. The respective exact masses should be deselected from the reverse analysis.

Additionally, the on-line MS/MS spectra databases with exact mass provision have to be further developed. At this stage there isn´t any feasible option that allows a time efficient molecular structure elucidation.

With an appropriate refining of the instrumental and software tools herby presented, non-targeted screening may be a reality in a STA context, in the near future. Moreover, the advantages that reverse search encloses will most probably serve other fields where analytical chemistry is of utility.

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6 APPENDIXES

Appendix I

Limit of detection, limit of fragmentation and lower therapeutic concentration for 198 representative pharmaco-toxicologically relevant compounds.

The 198 pure standards were present in the mixtures TOF mix 1 and TOF mix 2, having purities ≥ 98 %.

The concentrations considered in this sensitivity evaluation procedure ranged from 0.001 mg/Kg to 0.1 mg/Kg.

21 % did not fragment at concentrations below the LTC, 2 % were not detected within the considered concentrations and 4 didn´t provide fragments at the concentration of 0.1 mg/Kg.

Morphine was the most important miss on the provision of product ions.

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
1	1-(3-Chlorophenyl)piperazine (mCPP)	0,005	0,005	0.001	**0**	0	431	759	1618	4108	7283
2	2,5-Dimethoxy-4-idophenethylamine (2-CI)	0,002	0,005	N/A	0	79	568	968	2240	5325	9764
3	2,5-Dimethoxyamphetamine	0,002	0,02	0.02	0	79	522	875	**1844**	4022	7224
4	4-Bromo-2,5-Dimethoxyphenethylamine (2-CB)	0,005	0,01	N/A	0	0	91	388	882	2264	3983
5	4-FA (4-Fluoroamphetamine)	0,005	0,02	N/A	0	0	19	67	132	272	587
6	A29	0,002	0,02	N/A	0	79	0	0	425	948	1065
7	Acebutolol	0,001	0,02	0.2	196	845	3413	5786	12175	28114	**50360**
8	Acepromazine/Aceprometazine	0,001	<0.001	N/A	497	1316	3480	5807	12990	31352	58502
9	Acrivastine	0,001	<0.001	0.07	805	1484	3969	6716	14335	**33664**	62738
10	Alfentanil	0,001	<0.001	0.03	830	1581	4251	7434	**16388**	37256	70704
11	Alimemazine	0,001	<0.001	0.05	719	1326	3511	6224	13257	**33372**	64850
12	Almotriptan	0,001	0,002	0.05	422	791	3158	5256	11011	**26563**	49449
13	Alprazolam	0,001	0,01	0.005	29	100	**226**	488	968	2222	5379
14	Amfepramone	0,005	0,02	0.007	0	0	345	556	1861	4294	8052
15	Amiodarone	0,002	0,1	0.5	0	79	175	615	1483	5080	**20878**
16	Amisulpride	0,001	<0.001	0.1	441	1152	2447	5730	11409	25940	**56895**
17	Amitriptyline	0,001	0,005	0.05	382	940	2075	5054	9541	21836	**46942**
18	Amlodipine	0,001	0,02	0.003	199	494	1199	**2372**	4606	12250	19006
19	Amphetamine	0,01	0,05	0.02	0	0	0	15	**0**	162	319
20	Aripiprazole	0,001	0,002	0.15	288	629	1491	3587	7176	**16801**	35595
21	Atenolol	0,001	0,02	0.1	111	257	554	1292	2422	5932	**12495**
22	Atropine	0,001	0,005	0.002	358	**942**	2017	4781	9325	21284	45600
23	Baclofen	NA	NA	0.08	0	0	0	0	0	**0**	0
24	Benzfetermine	0,001	0,002	0.025	193	552	1514	2374	**5600**	13808	26056
25	Benzocaine	0,1	0,1	N/A	0	0	0	0	0	0	489

Legends: = Fragmentation / = No fragmentation / **XXXX** = LTC

Figure 6.I-6.1 *LOD, LOF and LTC for 25 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
26	Benzoylecgonine	0,001	0,005	0.1	140	339	743	1704	3506	7884	**17497**
27	Biperiden	0,001	0,002	0.05	808	1367	3536	6169	13512	**32102**	62019
28	Bisoprolol	0,001	0,002	0.01	375	1101	2804	**4811**	10131	22982	40550
29	Bromazepam	0,05	0,05	0.05	0	0	0	0	49	**146**	246
30	Bupivacaine/levobupivacaine	0,001	<0.001	0.25	397	987	2195	5117	9747	21801	**47519**
31	Buprenorphine	0,001	0,02	0.0005	**235**	579	1475	2972	5781	18643	31471
32	Buspirone	0,001	0,002	0.01	**526**	1729	4504	8154	17520	41416	78020
33	Caffeine	0,001	0,001	2	26036	25890	24146	25254	26323	24216	**26143**
34	Carbamazepine	0,001	<0.001	N/A	150	335	736	1729	3329	7508	**16323**
35	Carvedilol	0,002	0,005	N/A	0	**323**	1908	3216	6065	14305	26397
36	Cathine (Norpseudoephedrine)	0,02	0,1	0.71	0	0	0	0	8	16	**99**
37	Cathinone	0,005	0,02	0.08	0	0	19	89	159	347	**801**
38	Cetirizine	0,001	>0.1	0.02	257	453	738	1825	**3478**	7777	17596
39	Chlordiazepoxide	0,001	0,005	0.4	58	168	351	215	1617	3922	**8435**
40	Chlordiazepoxide, demetyl-	NA	NA	0.4	0	0	0	0	0	0	0
41	Chlorphen(ir)amine	0,001	<0.001	0.03	344	741	1969	3209	**7314**	16777	32110
42	Chlorpromazine	0,001	0,002	0.03	409	815	2122	3705	**7854**	20900	40972
43	Chlorprothixene	0,001	0,005	0.02	256	616	1451	3562	**6991**	17182	36438
44	Citalopram, desmethyl	0,001	<0.001	0.05	459	1185	2484	5815	11073	25251	53601
45	Citalopram/Escitalopram	0,001	<0.001	0.05	525	1356	2850	7072	**13417**	29512	66162
46	Clobazam	0,005	0,005	0.03	0	0	495	741	**1588**	3840	7086
47	Clomethiazole	NA	NA	0.1	0	0	0	0	0	0	**0**
48	Clomipramine	0,001	0,002	0.02	293	710	1584	3881	7315	17734	**37973**
49	Clonazepam	0,005	0,02	0.004	0	**0**	46	151	266	635	1460
50	Clonazepam, 7-amino	0,001	0,005	0.004	70	**141**	341	683	1664	3665	4638

Legends: = Fragmentation / = No fragmentation / XXXX = LTC

Figure 6.I-6.2 *LOD, LOF and LTC for 25 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
51	Clonidine	0,005	0,1	0.001	**0**	0	225	399	1825	4212	12701
52	Clozapine	0,001	<0.001	0.1	346	820	1826	4448	8614	20275	**42287**
53	Cocaine	0,001	<0.001	0.05	386	943	2093	4981	9519	**21789**	47653
54	Codeine	0,001	0,02	0.03	212	496	1102	2520	**4935**	11034	22824
55	Codeine, Nor (N-Desmethylcodeine)	0,002	>0.1	0.03	0	281	770	1186	**2731**	6519	11332
56	Cyclizine	0,001	0,002	0.1	125	717	1918	3253	7184	16277	**31982**
57	Dextromethorphan	0,001	0,005	0.01	332	850	1861	**4269**	8274	18764	41328
58	Diazepam	0,001	0,02	0.1	112	263	569	1333	2625	6241	**14033**
59	Dihydrocodeine	0,001	0,005	0.03	374	726	1875	3238	**6694**	14924	26602
60	Diltiazem	0,001	<0.001	0.03	914	1847	4864	8639	**19070**	44321	84526
61	Dimethyltryptamine (DMT)	0,002	0,005	0.001	**0**	75	578	963	2153	5014	9357
62	Diphenydramine	0,001	<0.001	0.05	175	359	746	1765	3280	**6536**	11119
63	Dipyridamol	0,001	0,005	0.1	542	1470	3329	7660	15282	34035	**76007**
64	Donepezil	0,001	0,002	0.03	954	1827	4758	8194	**18531**	42834	80106
65	Dosulepin	0,001	0,005	0.02	418	976	2256	5189	**9948**	23200	49966
66	Doxazosin	0,001	<0.001	0.01	600	1142	3117	**5556**	11965	27822	50620
67	Doxepine	0,001	0,005	0.01	358	772	1750	**4074**	7952	18269	39846
68	Duloxetin	0,001	>0.1	0.03	6	17	94	226	**417**	1101	2081
69	EDDP	0,001	<0.001	0.05	571	1098	2962	5086	10974	**26979**	35677
70	Eletriptan	0,001	0,002	0.06	867	1731	4433	7640	16026	**38241**	73036
71	Ephedrine	0,001	0,002	0.02	84	184	414	794	**1718**	3666	1065
72	Fenoterol	0,01	0,1	0.001	**0**	0	0	596	0	571	1003
73	Fentanyl	0,001	0,002	0.003	**416**	1012	2281	5515	10484	23700	51264
74	Fexofenadine	0,001	<0.001	0.3	398	755	2097	3753	7970	17728	**31988**
75	Flecainide	0,001	<0.001	0.2	1124	2280	6141	10494	22074	50757	**94481**

Legends: = Fragmentation / = No fragmentation / **xxxx** = LTC

Figure 6.I-6.3 *LOD, LOF and LTC for 25 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
76	Fluconazole	0,002	0,01	1	0	77	147	330	718	1506	**3408**
77	Flumazenil	0,005	0,005	0.01	0	0	525	**914**	2091	4671	8745
78	Flunitrazepam	0,001	0,01	0.005	102	209	**524**	1011	2416	5464	7002
79	Flunitrazepam, 7-amino	0,001	0,01	0.005	207	510	**1090**	2599	5067	11778	25490
80	Fluoxetine	0,001	>0.1	0.12	294	722	1762	3798	7419	18387	**36508**
81	Fluoxetine, demethyl	0,002	>0.1	0.12	0	12	123	179	329	1165	**1675**
82	Flupentixol	0,001	0,005	0.001	**340**	755	1768	4106	8408	21239	46497
83	Fluvoxamine	0,005	0,02	0.06	0	0	236	1388	3130	**8309**	15210
84	Formoterol	0,001	0,05	N/A	76	432	1185	1950	4152	8971	15263
85	Gabapentin	0,02	0,1	0.5	0	0	0	0	97	215	**466**
86	Haloperidol	0,001	<0.001	0.005	429	1048	**2418**	5824	10739	25936	53967
87	Hydrocodone	0,002	0,001	0.01	0	223	920	**1537**	3336	7699	14023
88	Hydromorphone	0,002	0,002	0.005	0	84	**770**	1186	2731	6519	11332
89	Hydroxyzine	0,001	<0.001	0.05	416	1000	2263	5135	9869	**22497**	49309
90	Imipramine	0,001	0,005	0.05	348	839	1931	4517	8628	**19818**	43133
91	Isocarboxazid	0,01	0,05	N/A	0	0	0	93	552	1512	2753
92	Ketamine	0,001	0,005	1	148	358	783	1920	3580	8241	**18722**
93	Ketamine, Nor	0,005	0,01	1	0	0	68	317	129	1342	**3063**
94	Ketobemidon	0,001	0,01	0.01	306	718	1606	3649	**6998**	15461	31588
95	Labetalol	0,002	0,005	0.03	0	142	905	1513	**3198**	7176	12256
96	Lamotrigine	0,001	0,05	1	167	429	921	2222	4209	9828	**20372**
97	Lercanidipine	0,001	<0.001	N/A	869	1523	4479	7294	16114	44004	91499
98	Levetiracetam	0,002	0,05	3	0	119	226	488	965	2028	**3839**
99	Levomepromazine	0,001	0,002	0.005	418	1039	**2375**	5641	10637	25670	54099
100	Levorphanol/Dextrorphan	0,001	0,002	0.007	156	957	**2557**	4161	9169	21466	39819

Legends: = Fragmentation / = No fragmentation / **XXXX** = LTC

Figure 6.I-6.4 *LOD, LOF and LTC for 25 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by a bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
101	Lidocaine	0,001	0,005	1	195	486	996	2399	4773	10108	**22515**
102	Loperamide	0,001	<0.001	N/A	**429**	1068	2417	6068	11907	27050	60391
103	Loratidine	0,001	<0.001	0.001	**451**	850	2238	3726	8267	19674	38650
104	Lorazepam	0,05	0,005	0.02	0	0	0	0	**0**	406	789
105	Lormetazepam	0,02	0,002	0.002	0	**0**	0	0	552	1346	2598
106	Losartan	0,005	0,01	0.2	0	0	377	546	1177	2576	**5138**
107	LSD	0,001	0,002	0.0005	**831**	1586	4213	7250	15440	35859	70301
108	MDA	0,001	0,01	0.4	16	54	78	144	253	523	**1019**
109	MDEA	0,002	0,002	0.4	0	368	949	1556	3377	7809	**14850**
110	MDMA	0,001	0,005	0.4	94	196	404	973	1818	3960	**7767**
111	MDPV	0,001	<0.001	N/A	562	1063	2868	4908	10767	25677	49879
112	Melperone	0,001	<0.001	0.03	456	904	2448	4080	**9158**	21803	40783
113	Meperidine/Pethidin	0,001	0,002	0.1	498	961	2388	4125	9147	21382	**40727**
114	Mepivacaine	0,001	0,002	0.4	395	766	2054	3520	7668	17340	**33118**
115	Mepyramine	0,001	<0.001	N/A	572	1216	0	1682	3569	24148	46850
116	Mescaline	0,02	0,02	1.5	0	0	0	0	416	1003	**1900**
117	Methadone	0,001	<0.001	0.05	474	1129	2545	6110	11520	**26261**	58172
118	Methamphetamine	0,001	0,01	0.1	33	95	204	438	843	1770	**3334**
119	Methaqualone	0,001	0,002	1	108	547	1440	2367	5541	12747	**24380**
120	Methotrexate	NA	NA	0.04	0	0	0	0	**0**	0	0
121	Methoxymethamphetamine (PMMA)	0,005	0,005	N/A	0	0	548	941	2015	4396	7889
122	Methylphenidate	0,001	0,002	0.01	225	545	1209	**2997**	5587	12334	27616
123	Metoclopramide	0,001	<0.001	0.05	261	649	1397	3321	6604	**14589**	32219
124	Metoprolol	0,001	0,01	0.035	345	809	1803	3979	**7694**	16592	32491
125	Mianserine	0,001	<0.001	0.015	339	826	1682	**4254**	7985	17894	36756

Legends: = Fragmentation / = No fragmentation / **XXXX** = LTC

Figure 6.I-6.5 *LOD, LOF and LTC for 25 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by a bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
126	Midazolam	0,001	0,002	0.04	231	602	1304	3327	**6368**	13976	29730
127	Mirtazapine	0,001	<0.001	0.03	271	659	1434	3523	**6674**	15274	33591
128	Moclobemide	0,001	<0.001	0.3	394	723	1955	3218	7001	16443	**30572**
129	Morphine	0,005	>0.1	0.01	0	0	77	**239**	409	939	2159
130	Morphine, 6-monoacetyl	0,001	0,01	0.01	260	599	1353	3107	6066	13072	25795
131	m-Trifluorophenylpiperazine (TFMPP)	0,002	0,002	N/A	0	375	991	1634	**3550**	8629	15662
132	Naloxone	0,001	0,01	0.01	26	52	222	**853**	1709	3882	8372
133	Naltrexone	0,002	0,002	0.003	0	**429**	1121	1881	3829	9197	17248
134	Nitrazepam	0,005	0,05	0.03	0	0	28	**123**	207	501	1107
135	Nitrazepam, 7-amino	0,001	0,01	0.03	83	252	574	**1306**	2545	6014	11846
136	Nordiazepam	0,002	0,1	0.02	0	26	97	222	**410**	922	2101
137	Noscapine	0,001	<0.001	0.02	847	1723	4620	7864	**16747**	39849	76748
138	Olanzapine	0,001	<0.001	0.02	240	545	1111	3079	**6201**	13386	30619
139	Orphenadrine	0,001	<0.001	0.1	157	370	819	1861	3670	8028	**16418**
140	Oxazepam	0,005	0,05	0.2	0	0	55	121	229	506	**1081**
141	Oxcarbazepine	0,002	0,02	10	0	44	130	263	500	1200	**2557**
142	Oxycodone	0,001	0,002	0.005	109	260	**599**	1292	1897	5711	10530
143	Oxymorphone	0,005	0,02	N/A	0	0	313	491	1004	2365	4406
144	Paracetamol	0,001	0,1	5	420	446	223	504	607	543	**1199**
145	Paroxetine	0,001	0,01	0.01	296	727	1824	**3693**	6809	18120	30756
146	Perphenazine	0,002	0,002	0.001	**0**	644	1648	3309	6595	17314	34646
147	Phenazepam	0,02	0,05	0.02	0	0	0	0	**129**	775	1467
148	Phencyclidine (PCP)	0,001	0,002	0.01	206	319	872	**1500**	3304	7586	14025
149	Phentermine	0,1	>0.1	0.03	0	0	0	0	**0**	0	93
150	Pimozide	0,002	0,01	0.004	0	**133**	1222	2156	4582	12016	22755

Legends: = Fragmentation / = No fragmentation / **XXXX** = LTC

Figure 6.I-6.6 *LOD, LOF and LTC for 25 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by a bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
151	Pindolol	0,002	0,05	0.02	0	89	870	1423	**3011**	7290	12458
152	Pizotifen	0,001	<0.001	0.007	696	1305	**3429**	6048	13448	31763	60092
153	Pregabalin	0,02	>0.1	2	0	0	0	0	31	66	**169**
154	Prochlorperazine	0,05	0,1	0.01	0	0	0	**0**	0	369	1190
155	Procyclidine	0,001	<0.001	0.08	657	1270	3297	5853	12517	**30268**	57404
156	Promethazine	0,001	0,002	0.05	290	710	1640	**3801**	7255	17209	36929
157	Propafenone	0,001	0,002	0.04	680	1847	4894	8127	**18016**	42154	79335
158	Propoxyphen	0,001	0,01	0.05	135	238	2057	3611	7846	**18089**	34377
159	Propranolol	0,001	0,01	0.02	326	793	1816	4216	**7880**	17382	33711
160	Pseudoephedrine	0,005	0,005	0.05	0	0	309	480	1160	**2488**	4381
161	Psilocine	0,05	0,05	0.008	0	0	**0**	0	0	1283	2135
162	Quetiapine	0,001	<0.001	0.1	572	1430	3220	7664	14638	32085	**67461**
163	Quinidine	0,001	0,005	1 .0	387	715	2853	4733	10204	23719	**41860**
164	Quinine	0,001	0,01	1.0	499	1038	2067	4846	9294	21034	**43336**
165	Reboxetine	0,001	0,002	0.06	372	676	1850	3252	6606	**15214**	27155
166	Remifentanil	0,001	0,002	0.02	600	1186	2971	5290	**10738**	25315	48368
167	Risperidone	0,001	<0.001	0.006	566	1311	**3004**	6963	13043	30397	67450
168	Risperidone, hydroxy	0,001	<0.001	0.006	539	1285	**2925**	7021	13297	30120	65317
169	Ritalin acid	0,02	0,02	0.1355	0	0	0	0	336	755	**1386**
170	Salbutamol	0,005	0,005	0.004	0	**0**	445	776	3569	3868	6856
171	Salmeterol	0,001	<0.001	<0.001	**429**	799	2106	3603	7096	15482	25654
172	Scopolamine	0,001	0,002	0.0001	**546**	1044	2797	4791	10327	22963	42828

Legends: = Fragmentation / = No fragmentation / **xxxx** = LTC

Figure 6.I-6.7 *LOD, LOF and LTC for 22 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by a bold and underlining numbering.*

	PTRC pure standard	LOD (mg/Kg)	LOF (mg/Kg)	LTC (mg/Kg)[21]	0.001mg/Kg	0.002mg/Kg	0.005mg/Kg	0.01mg/Kg	0.02mg/Kg	0.05mg/Kg	0.1mg/Kg
176	Sildenafil	0,002	0,05	0.05	0	0	0	0	1261	**4601**	8799
177	Sotalol	0,001	0,002	0.5	75	367	979	1704	3554	7860	**13730**
178	Sulpiride	0,001	<0.001	0.05	634	1203	3105	5253	11177	**26818**	50483
179	Sumatriptan	0,001	0,005	0.018	131	749	2019	**3561**	7486	16875	30947
180	Temazepam	0,001	0,01	0.02	33	90	299	570	1062	**2312**	4822
181	Terbutaline	0,005	0,01	0.001	**0**	0	90	208	372	971	2029
182	Tolbutamide	0,05	0,1	0.007	0	0	**0**	0	0	471	910
183	Topiramate	0,01	>0.1	2	0	0	0	8	30	101	**274**
184	Tramadol	0,001	0,1	0.1	502	996	1792	4255	8164	17291	**37567**
185	Tramadol, O-desmethyl	0,001	0,05	0.1	262	542	1059	2542	4869	10861	**23374**
186	Triazolam	0,002	0,005	0.002	0	**103**	1864	1276	2772	6560	12294
187	Trimipramine	0,001	0,002	0.01	618	1149	3084	**5303**	9002	28237	56006
188	Varenicline	0,01	0,02	0.004	0	**0**	0	610	8645	2986	5546
189	Venlafaxine	0,001	0,005	0.1	252	614	1376	3223	6315	14124	**31371**
190	Venlafaxine, O-demethyl	0,001	0,005	0.1	240	603	1281	3070	6075	12973	**26863**
191	Verapamil	0,001	<0.001	0.01	852	1707	4625	**8055**	17178	40474	74569
192	Xylometazoline	0,001	0,005	N/A	209	1295	3287	5927	13186	30897	59858
193	Zaleplon	0,002	0,005	0.001	**0**	377	985	1747	3853	8954	16676
194	Ziprasidon	0,001	0,005	0.05	220	513	1231	2763	5436	**13179**	24775
195	Zolmitriptan	0,002	0,005	0.007	0	691	**1811**	3021	6625	15119	29621
196	Zolpidem	0,001	0,001	0.08	332	872	1944	4503	8625	**19982**	42777
197	Zopiclone	0,001	0,05	0.01	21	46	0	**176**	385	3229	7141
198	Zuclopenthixol	0,001	0,01	0.004	265	**645**	1535	3518	6924	17387	36856

Legends: = Fragmentation / = No fragmentation / **xxxx** = LTC

Figure 6.I-6.8 *LOD, LOF and LTC for 23 PTRC´s pure standards. LOD is defined as soon as a value for concentration is provided, LOF is determined as soon a green background is provided; LTC is stipulated by a bold and underlining numbering.*

Appendix II

"Excluded" library listing the 34 compounds not considered in Filter I.

Chemical excluded	"Summary plot" report	Toxicological information [21]
Adrenalone	Present in all blood samples, including in blind blood.	Adrenergic agonist used as a topical vasoconstrictor and hemostatic. Non-PTRC.
Allopurinol	Present in almost all blood samples, including in blind blood.	Xanthine oxidase inhibitor for treatment of hyperuricemia. Non-PTRC.
Benalaxyl	Present in almost all blood samples, including in blind blood.	Fungicide. Rarely toxic. Not appropriate for analysis.
Caffeine	Present in almost all blood samples, including in blind blood.	Mild central nervous system stimulant. Non-PTRC.
Carbidopa	Present in almost all blood samples, including in blind blood.	Employed in the treatment of Parkinson´s disease by inhibiting the peripheral metabolism of levodopa. Non-PTRC.
Cefalexin	Present in 10% of samples, including in blind blood. Only as an Na^+ Adduct.	Cephalosporin antibiotic. Good alternative to penicillins to treat patients with penicillin hypersensitivity. Non-PTRC.
Clindamycin	Present in 10% of samples, including in blind blood. Only as an Na+ adduct.	Lincosamide antibiotic used to treat infections with anaerobic bacteria and some protozoal diseases, such as malaria. Not appropriate for analysis.
Corticosterone	Present in all blood samples, including in blind blood.	Endogenous steroid hormone. Non-PTRC.
Cortisol	Present in all blood samples, including in blind blood.	Endogenous steroid hormone. Non-PTRC.
Cortisone	Present in all blood samples, including in blind blood.	Endogenous corticosteroid released by adrenal gland in response to stress. Non-PTRC.
Cotinine	Present almost all blood samples, including in blind blood.	Alkaloid metabolite of Nicotine. Rarely toxic.
Demeton-S-Methyl Sulphone	Present in 30% of samples, including in blind blood.	Acaricide and Insecticide. Toxicological relevant.
Diprophylline	Present in 20% of samples, including in blind blood.	Xanthine derivate with bronchodilator and vasodilator effects. Used for treatment of asthma, bronchitis. Non-PTRC.
Drospironone	Identified in 10% of samples, including in blind blood.	Synthetic hormone used in birth control pills. Non-PTRC.
Ecgonine methyl ester	Present in almost all blood samples, including in blind blood.	Compound that result from the consumption of cocaine and alcohol within a short time spam. Not suited for analysis.
Etamiphyline	Present in 50% of samples, including in blind blood.	Xanthine intended for use as anti-asthma agent. Non-PTRC.

Appendix II (continue)

Chemical excluded	"Summary plot" report	Toxicological information [21]
Etamivan	Present in all samples, including in blind blood.	Respiratory stimulant drug used for treatment of barbiturate overdose and chronic obstructive pulmonary disease. Rarely toxic. Not suited for analysis.
Hydroxyprogesterone	Present in 10% of samples, including in blind blood.	Endogenous steroid hormone produced during the synthesis of glucocorticoids and sex steroids. Non-PTRC.
Lovastatin	Present in all blood samples, including in blind blood.	Antihyperlipidermic agent. Non-PTRC.
Meropenem	Present in 30% of samples, including in blind blood.	Ultra-broad spectrum injectable antibiotic used to treat a wide variety of infections. Very effective against enterobacteriaceae but not so much against gram +ve species. Not appropriate for analysis.
Methylprednisolone	Present in 20% of samples, including in blind blood.	Synthetic glucocorticoid or corticosteroid with anti-inflammatory effects. Over usage may lead to hyperglycemia and hypertension. Not appropriate for analysis.
Metipranolol	Present in all blood samples, including in blind blood.	Beta blocker used in eye drops to treat glaucoma. Non-PTRC.
Naftifine	Present in 20% of samples, including in blind blood. Only as a Na^+ adduct.	Antifungal drug. Precise mechanism of action unknown. Not appropriate for analysis.
Nateglinide	Present in all samples, including in blind blood.	Drug for treatment of Type 2 Diabetes. Non-PTRC.
Nicotinamide	Present in all blood samples, including in blind blood.	Amine of nicotinic acid (Vit. B3/niacin). Non-PTRC.
Norelgestromin	Present in 10% of samples, including in blind blood. Only as a Na^+ adduct	Progestin used in the contraceptive patch called Evra. Non-PTRC.
Phenylephrine	Present in more than 30% of samples, including in blind blood.	Selective Alpha-drenergic receptor agonist used primarily as a decongestant, then as a pupil dilator and lastly, to increase blood pressure. Non-PTRC.
Simvastatin	Present in almost in all blood samples, including in blind blood.	Reductase inibitor related to lovastatin. antihyperlipidermic agent. Non-PTRC.
Spiromesifen	Present in 40% of samples, including in blind blood.	New generation insecticide against the whitefly (specie that attack crops). Not appropriate for analysis.
Theobromine	Present in all blood samples, including in blind blood.	Bitter alkaloid of the cacao plant. Non-PTRC.

Appendix II (continue)

Chemical excluded	"Summary plot" report	Toxicological information [21]
Theophyline	Present in all blood samples, including in blind blood.	Related to caffeine. It is a bronchodilator in asthma treatment. Non-PTRC.
Tryptophan	Present in all blood samples, including in blind blood.	Endogenous aminoacid. Non-PTRC.
Urapidil	Present in 10% of samples, including in blind blood.	Sympatholytic antihypertensive drug that acts as an α_1-adrenoceptor antagonist and as an 5-HT1A receptor agonist. Non-PTRC.
Valaciclovir	Present in 10% of samples, including in blind blood. Only as a Na^+ adduct.	Antiviral drug used in the management of herpes simplex, herpes zoster (Shingles) and herpes B. Non-PTRC.
Veralipride	Present in 20% of samples, including in blind blood.	Benzamide neuroleptic drug employed in the treatment of vasomotor symptoms associated with the menopause. Non-PTRC.

Appendix III

Table containing all targeted screening results.

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM2938	Kidney disease	Levomepromazine	UP	0.024	
		Enalapril	**FN**	0.001	LTC Above
		Hydrocodone	UP	Negative	
		Codeine	UP	0.16	
		Codeine, glucuronide-	UP	Negative	
		Morphine	**FN**	**0.04**	**LTC Below**
TIM2943	Drowning and heart disease	Amlodipine	UP	0.057	
TIM2945	Traffic (*antemortem*)				
TIM2946	Cause of death not found; Drug addict	Benzoylegonine	UP	Negative	
		Clonazepam, 7-amino-	**UP**	**Negative**	Clonazepam metabolite
		Diazepam	UP	0.039	
		EDDP	UP	Negative	
		Bromazepam	**FN**	0.013	LTC Above
		Cocaine	**FN**	0.001	LTC Above
		Haloperidol	UP	Negative	
		Methadone	UP	0.35	
		Diazepam, demethyl-	UP	0.028	
		Clonazepam	**FN**	0.056	LTC Below / metabolite present
TIM2949	Traffic (*antemortem*)	Cetirizine	UP	0.12	
		Diazepam, desmethyl-	**FN**	0.002	LTC Above
		Paracetamol	UP	Negative	
		Venlafaxine	UP	0.46	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM2949 (continue)	Traffic (*antemortem*) (continue)	Venlafaxine, O-desmethyl	UP	0.096	
		Zolpidem metabolite 1	UP	Negative	
		Zolpidem metabolite 2	UP	Negative	
		Chlorprothixene, desmethyl-	UP	0.073	
		Chlorprothixene	UP	0.17	
		Zolpidem	UP	0.16	
TIM2952	Methadone poisoning	Bromazepam	UP	0.34	
		EDDP	UP	Negative	
		Methadone	UP	1.5	
		Mirtazapine	UP	0.04	
		Mirtazapine, 8-hydroxy-	UP	Negative	
		Mirtazapine, N-desmethyl-	UP	Negative	
TIM2955	Methadone poisoning	Benzoylecgonine	UP	0.11	
		Cetirizine	UP	Negative	
		Cocaine	**UP**	**Negative**	
		Chlorprothixene, desmethyl-	UP	0.057	
		Dextromethorphan	UP	Negative	
		EDDP	UP	Negative	
		Hydroxizine	UP	0.002	
		Lidocaine	UP	Negative	
		Methadone	UP	0.46	
		Morphine	**FN**	0.034	LTC Below / related compound identified
		6-MAM-	**UP**	**Negative**	Same properties as morphine

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM2955 (continue)	Methadone poisoning (continue)	Morphine, dihydro-	UP	Negative	
		Noscapine	UP	Negative	
		Papaverine	UP	Negative	
		Paracetamol	UP	Negative	
		Quinine	UP	Negative	
		Solifenacin	UP	Negative	
		Yohimbine	UP	Negative	
		Chlorprothixene	UP	0.13	
		Codeine	UP	0.01	
TIM2956	Violence (*antemortem*)	Benzoylecgonine	**FN**	0.01	LTC Above
		Paracetamol	UP	Negative	
TIM2964	Traffic (*antemortem*)				
TIM2965	Traffic (*antemortem*)				
TIM2967	Lung disease	Metoprolol	UP	0.019	
		Metoprolol, hydroxyl-	UP	Negative	
		Primidone	UP	3.0364	
		Terbutaline	UP	0.01	
TIM2968	Heart disease				
TIM2970	Sexual assault (*antemortem*)	Citalopram, desmethyl-	UP	10	
		Citalopram, didesmethyl-	UP	Negative	
		Citalopram/ Escitalopram	UP	7.2	
TIM2971	Violence (*antemortem*)				
TIM2972	Traffic (*antemortem*)				

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM2976	Methadone poisoning	EDDP	UP	Negative	
		Methadone	UP	0.4	
		Diazepam, desmethyl-	UP	2.2	
		Demoxepam	**FN**	2.5	LTC Below / Parent drug present
		Promethazine	**FN**	0.0005	LTC Above
		Oxazepam	UP	0.017	
		Paracetamol	UP	Negative	
		Tramadol	UP	0.51	
		Tramadol, N-desmethyl-	UP	Negative	
		Tramadol, O-desmethyl-	UP	0.13	
		Chlordiazepoxide	**UP**	1.4	Demoxepam parent drug
TIM2978	Methadone poisoning	Citalopram/ Escitalopram	UP	0.34	
		Citalopram, desmethyl-	UP	0.28	
		Citalopram, didesmethyl-	UP	Negative	
		Clonazepam, 7-amino-	UP	0.21	
		Chlorprothixene, desmethyl-	UP	0.08	
		EDDP	UP	Negative	
		Methadone	UP	1.3	
		Paracetamol	UP	Negative	
		Quetiapine	UP	0.15	
		Chlorprothixene	UP	0.092	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM2982	2nd degree burn and carbon monoxide poisoning	Lidocaine	UP	Negative	
		Quetiapine	UP	0.006	
TIM2998	Methadone poisoning	Clonazepam, 7-amino-	UP	0.23	
		EDDP	UP	Negative	
		Methadone	UP	0.46	
		Nitrazepam, 7-amino-	UP	0.009	
		Paracetamol	UP	Negative	
TIM3001	Choking	Chlordiazepoxide	**UP**	0.1	Demoxepam parent drug
		Doxepine	UP	Negative	
		Demoxepan	**FN**	0.87	LTC Below / parent drug present
		Oxazepam	UP	0.085	
		Oxazepam, glucuronide-	UP	Negative	
		Promathezine	UP	0.009	
		Promethazine, sulphoxide-	UP	Negative	
		Diazepam, desmethyl-	UP	0.84	
		Amlodipine	UP	0.033	
TIM3002	Heart disease	Diazepam, desmethyl-	**FN**	0.002	LTC Above
		Paracetamol	UP	Negative	
TIM3003	Cause of death Not found. (High alcohol level)	Diazepam, desmethyl-	**FN**	0.013	LTC Above
		Chlorprothixene	**FN**	0.0001	LTC Above

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3004	Internal bleeding	Ketobemidone	UP	Negative	
		Paracetamol	UP	Negative	
		Roxithromycin	UP	Negative	
		Tramadol	UP	0.15	
		Tramadol, N-desmethyl-	UP	Negative	
		Tramadol, O-desmethyl-	UP	0.05	
TIM3005	Heart disease	Cinchonine	UP	Negative	
		Hydroquinidine	UP	Negative	
		Paracetamol	UP	Negative	
		Propranolol	UP	0.14	
		Quinine	UP	0.5	
		Warfarin	UP	0.61	
		Citalopram	**FN**	0.003	LTC Above
TIM3006	Traffic (*antemortem*)				
TIM3010	Heart disease	Cinchonine	UP	Negative	
		Quinine	UP	Negative	
		Sertraline	UP	0.015	
		Zopiclone	UP	0.011	
		Zopiclone, desmethyl-	UP	Negative	
TIM3012	Assault victim (*antemortem*)	Benzoylecgonine	UP	0.014	
		Cocaine	**FN**	0.003	LTC Above
		Hydroxyzine	UP	Negative	
		Lidocaine	UP	Negative	
		Metronidazole	UP	Negative	
		Morphine	**FN**	**0.035**	**LTC Below**
		Remifentanil	UP	Negative	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3014	Liver disease	Chlordiazepoxide	**UP**	0.65	Demoxepam parent drug
		Diazepam, desmethyl-	**FN**	0.01	LTC Above
		Demoxepam	**FN**	0.31	LTC Below / parent drug present
		Paracetamol	UP	1.0	
TIM3017	Methadone Poisoning	Citalopram, desmethyl-	UP	0.33	
		Citalopram, didesmethyl-	UP	Negative	
		Citalopram/ Escitalopram	UP	1.2	
		EDDP	UP	Negative	
		Methadone	UP	2.4	
		Oxazepam	UP	0.07	
		Paracetamol	UP	30	
TIM3018	Heart disease	Amlodipine	UP	Negative	
		Citalopram, didesmethyl-	UP	Negative	
		Nortriptyline	UP	0.17	
		Risperidone, hydroxyl-	UP	Negative	
		Amitriptyline	UP	0.13	
		Citalopram/ Escitalopram	UP	0.32	
		Citalopram, desmethyl-	UP	0.19	
TIM3019	Burned	Alfentanil	UP	Negative	
		Atenolol	UP	Negative	
		Citalopram/ Escitalopram	UP	0.52	
		Citalopram, desmethyl-	UP	0.26	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3019 (continue)	Burned (continue)	Citalopram, didesmethyl-	UP	Negative	
		Midazolam	UP	Negative	
		Paracetamol	UP	Negative	
		Fentanyl	UP	Negative	
TIM3026	Heart disease				
TIM3027	Violence (*antemortem*)	Ibuprofen	**FN**	5.0	Non-PTRC
		Paracetamol	UP	1.0	
		Tramadol	UP	0.16	
		Tramadol, O-desmethyl-	UP	0.057	
		Tramadol, N-desmethyl-	UP	Negative	
TIM3033	Violence (*antemortem*)	Nortriptyline	UP	0.009	
		Nitrazepam, 7-amino-	UP	0.036	
		Amitriptyline	UP	0.02	
		Nitrazepam	UP	0.046	
TIM3034	Violence (*antemortem*)	Lidocaine	UP	Negative	
		Midazolam	UP	Negative	
		Mono-ethylglycinexylidide	UP	Negative	
		Paracetamol	UP	Negative	
		Metronidazole	UP	Negative	
TIM3037	Internal bleeding	Atropine	UP	Negative	
TIM3038	Fat embolism syndrome	Ciprofloxacin	UP	Negative	
		Lidocaine	UP	Negative	
		Morphine	UP	2.5	
		Oxycodone	UP	0.004	
		Oxycodone, desmethyl-	UP	Negative	
		Paracetamol	UP	Negative	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3038 (continue)	Fat embolism syndrome (continue)	Bupivacaine/ Levobupivacaine	UP	Negative	
		Diazepam	**FN**	0.005	LTC Above
TIM3040	Violence (*antemortem*)	Benzoylecgonine	UP	0.001	
TIM3041	Violence (*antemortem*)	Mirtazapine	UP	0.043	
		Mirtazapine, 8-hydroxy-	UP	Negative	
		Mirtazapine, N-desmethyl-	UP	Negative	
TIM3044	Traffic (*antemortem*)	Amfetamine	UP	0.088	
		Benzoylegonine	UP	0.16	
		Bromazepam	**FN**	0.01	LTC Above
		Diazepam	UP	1.1	
		Fenoterol	UP	Negative	
		Diazepam, desmethyl-	UP	1.2	
		Morphine	**FN**	0.005	LTC Above
		Oxazepam	UP	0.2	
		Oxycodone, desmethyl-	UP	Negative	
		Oxycodone	UP	0.047	
		Paracetamol	UP	Negative	
		Temazepam	UP	0.2	
TIM3045	Morphine poisoning	Benzoylegonine	UP	0.18	
		Cocaethylene	UP	Negative	
		Cocaine	UP	0.04	
		Codeine	UP	0.015	
		Levamisole/ Tetramisole	UP	Negative	
		Lidocaine	UP	Negative	
		Morphine	UP	0.12	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3045 (continue)	Morphine poisoning (continue)	Noscapine	UP	Negative	
		Amphetamine	**FN**	0.002	LTC Above
		Papaverine	UP	Negative	
		Paracetamol	UP	Negative	
		Phenacetine	UP	Negative	
TIM3046	External bleeding	Olanzapine	UP	0.1	
		Olanzapine, N-desmethyl-	UP	Negative	
		Risperidone, hydroxyl-	UP	0.05	
TIM3051	Cause of death not found; High levels of zuclopentixol	Clonazepam, 7-amino-	UP	0.026	
		Paracetamol	UP	90	
		Zuclopenthixol	UP	0.032	
TIM3052	Phenobarbital poisoning	Benzoylecgonine	UP	0,002	
		Citalopram, desmethyl-	UP	0,092	
		Citalopram, didesmethyl-	UP	Negative	
		Clonazepam	UP	0,002	
		Clonazepam, 7-amino-	UP	0,039	
		Diazepam	UP	0,11	
		Diphenydramine	UP	0,1	
		Phenobarbital	**FN**	**82**	**Barbituric**
		Methylphenidate	UP	0,006	
		Mirtazapine	UP	1,7	
		Mirtazapine, 8-hydroxy-	UP	Negative	
		Mirtazapine, N-desmethyl-	UP	Negative	
		Diazepam, desmethyl-	UP	0,03	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3052 (continue)	Phenobarbital poisoning (continue)	Oxazepam	**FN**	0.004	LTC Above
		Paracetamol	UP	1	
		Promethazine	UP	0,13	
		Promethazine, hydroxydesmethyl	UP	Negative	
		Promethazine, sulphoxide-	UP	Negative	
		Temazepam	UP	0,012	
		Alprazolam	UP	0,071	
		Citalopram/ Escitalopram	UP	0,18	
TIM3054	Shot in the head				
TIM3056	Internal bleeding	Enalaprilat	**FN**	0,014	LTC Above
		Metoprolol	UP	0,02	
		Metoprolol, hydroxy-	UP	Negative	
		Enalapril	**FN**	0,01	LTC Above
TIM3057	Internal bleeding	Acrivastine	UP	Negative	
		Quinine	UP	Negative	
TIM3059	Traffic				
TIM3060	Traffic (*antemortem*)				
TIM3061	Traffic (*antemortem*)	Cinchonine	UP	Negative	
TIM3062	Traffic (*antemortem*)				
TIM3063	Cause of death not found	Paracetamol	UP	Negative	

Appendix III (continue)

Case	Police / RKA Report	Compound	*UNIFI1.7™*	LIMS (mg/Kg)	FN evaluation
TIM3064	Inflammation of pancreas	Aripiprazole	UP	0,15	
		Lamotrigine	UP	0,9	
		Olanzapine	UP	0,001	
		Olanzapine, N-desmethyl-	UP	Negative	
		Paracetamol	UP	Negative	
		Terbutaline	UP	0,016	
TIM3068	Internal bleeding and Methadone poisoning	Ambroxol	UP	Negative	
		Bromhexine	UP	Negative	
		Codeine	UP	0,11	
		Codeine, glucuronide-	UP	Negative	
		Codeine, desmethyl-	UP	Negative	
		EDDP	UP	Negative	
		Hydrocodone	UP	Negative	
		Methadone	UP	1,9	
		Paracetamol	UP	Negative	
		Zopiclone	UP	0,064	
		Zopiclone, desmethyl-	UP	Negative	
TIM3069	Internal bleeding	Amisulpride	UP	0.26	
		Losartan	UP	Negative	